Current Aspects of Leukemia Inhibitory Factor (LIF) and its Signaling Pathways in Choriocarcinoma Cell Lines

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Dedicated to my family,
on both sides of the Atlantic

Dedicated to my grandmother Jandira,
who indirectly taught me the value of learning

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List of Abbreviations

γδT-Cells	Gamma Delta T-Cells
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CLB	Cell Lysis Buffer
CNF Ciliary Neurotrophic Factor	
DAPI	4,6-Diamidin-2-phenylindol-dihydrochlorid
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
dsRNA	Double-Stranded RNA
EGF	Epidermal Growth Factor
EPO	Erythropoietin
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
Fig	Figure
FSC	Forward Scatter
GBSS	Gey's Balanced Salt Solution
G-CSF	Granulocyte Colony-Stimulating Factor
GH	Growth Hormone
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GTD	Gestational Trophoblastic Disease
GTN	Gestational Trophoblastic Neoplasia
hCC	Human Choriocarcinoma Cell
hCG	Human Chorionic Gonadotropin
HGF	Hepatocyte Growth Factor
HGPRT	Hypoxanthine-Guanine Phosphoribosyltransferase
HLA-G	Human Leukocyte Antigen G
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
JAK	Janus Kinase
kDa	kilo Dalton
LIF	Leukemia Inhibitory Factor

MHC	Major Histocompatibility Complex
min	minute
miRNA	microRNA
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
NK-cells	Natural Killer Cells
PBS	Phosphate Buffered Saline
Ph-JAK1	Phosphorylated Janus Kinase 1
Ph-JAK2	Phosphorylated Janus Kinase 2
Ph-JAK3	Phosphorylated Janus Kinase 3
PHA	Phytohemagglutinin
PIAS	Protein Inhibitor of Activated STAT
PRL	Prolactin
PTP	Protein Tyrosine Phosphatase
R	Receptor
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic Acid
RNAi	RNA Interference
rpm	Rounds per Minute
RPMI	Roswell Park Memorial Institute Medium
RT-PCR	Reverse Transcription Polymerase Chain Reaction
Scr	Scrambled siRNA (siRNA with a non-genomic sequence)
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	Short / Small Hairpin RNA
siRNA	Small Interfering RNA
SOCS	Suppressor of Cytokine Signaling
SSC	Sideward Scatter
STAT	Signal Transducer and Activator of Transcription
TGF-β	Transforming Growth Factor-β
Th1/Th2	T-Helper Type1 / Type2
TNF	Tumor Necrosis Factor
TPO	Thrombopoietin
Tyr	Tyrosine

Summary

Introduction

Several parallels may be drawn between pregnancy and cancer. In the course of embryo implantation, trophoblast cells are able to proliferate and invade into maternal tissues, while a complex interaction with the maternal immune system ensures immune tolerance. Similarly, many tumors are able to grow invasively and mask themselves from the immune system. Many of the underlying mechanisms governing embryo implantation and tumor progression seem to lie in close proximity. Among them, the JAK/STAT pathway has been implicated in the transduction of a multitude of signals governing cell differentiation, proliferation, migration and apoptosis. Leukemia Inhibitory Factor (LIF) is a cytokine maximally expressed at the time of implantation that signals through the JAK/STAT pathway. Although its roles have not been completely understood, LIF is assumed to facilitate embryo implantation and regulate tumor invasion through the activity of STAT3. In this sense, this work intended to better clarify the role of LIF and the JAK/STAT pathway in choriocarcinoma cell lines, which are considered models for studying trophoblast physiology.

Methods

Different choriocarcinoma cell lines have been stimulated with LIF and IL-6 in order to investigate if these cytokines were associated with the tyrosine phosphorylation of STAT3, detected by Western blot. Considering that these cytokines are known to signal through the JAK/STAT pathway, Western blot has been performed to assess the constitutive expression and phosphorylation of three Janus kinases (JAKs) that might be involved in the LIF signaling pathway. Immunocytochemistry has been performed to validate some of these results. Additionally, this technique has been performed to assess the constitutive expression of the three Janus kinases in placental tissues of a healthy woman undergoing elective abortion. Once determined which Janus kinase had been expressed and phosphorylated upon LIF stimulation in the choriocarcinoma cell lines, a set of experiments aiming to silence kinase expression specifically has been performed. Trying to achieve this goal, different transfection reagents and siRNA concentrations have been tested for RNA interference targeting JAK1. Knock-down efficiency has been evaluated by Western blot. Finally, a set of functional tests has been performed to evaluate if LIF treatment and/or kinase silencing might modify cell behavior. In this sense, flow cytometry has been used to quantify cell proliferation, migration and invasion rates.

Results

As demonstrated by Western blot, LIF stimulation correlated with the phosphorylation of STAT3 in the choriocarcinoma cell lines, as opposite to IL-6. The constitutive expression and phosphorylation of JAKs were differently distributed among cells. JAR cells expressed JAK1 and JAK2 constitutively, and LIF stimulation induced phosphorylation of JAK1 in these cells. JEG-3 cells expressed JAK2 just to a marginal degree, and its phosphorylated form did not alter after LIF stimulation. Constitutive or activated JAK3 were not identified in both cell groups. Immunocytochemistry demonstrated similar results concerning the JAK isoforms constitutively expressed in these cells. Similarly, JAK1 and JAK2, but not JAK3, were identified in placental tissues, mainly in the villous trophoblast.

As JAK1 was demonstrated to be constitutively expressed and phosphorylated after LIF stimulation in JAR cells, this isoform was chosen as target in the experiments of RNA interference. Maximal silencing rates (about 65% JAK1 knock-down) were achieved using Oligofectamine for transfection of the siRNA. In the functional experiments, LIF stimulation correlated with a significant increase in the proliferation rate of JAR cells. JAK1 knock-down induced a decrease in migratory cell behavior.

Discussion

The results of this work bring some help to understand the possible actors playing a role in cytokine signaling within the JAK/STAT pathway in choriocarcinoma cells. This pathway represents the principal signaling mechanism for a wide group of growth factors and cytokines in animals. The constitutive expression of three JAKs has been revealed in different choriocarcinoma cell lines and placentary tissues, evidencing similar profiles. Furthermore, it has been investigated how these JAK isoforms are influenced by LIF, a cytokine considered indispensable during placentation in several species and that is involved in regulation of tumor progression. LIF has also been demonstrated to induce STAT3 activation in all choriocarcinoma cell lines investigated in this work. STAT3 is implicated in neoplastic cell behavior, playing a role in cell motility and invasive growth. In this sense, it was demonstrated that LIF influences the behavior of JAR cells, increasing its proliferation rate. Reduction of JAK1 expression, achieved by RNA interference, correlated with altered cell behavior, posing the JAK/STAT pathway as a possible target of molecular based therapies. The clarification of the multitude of factors and signaling pathways regulating cell proliferation and invasion, both in the physiological and pathological settings, may lead to the development of new therapies targeting cancer and pregnancy related pathologies.

Zusammenfassung

Einleitung

Zwischen einer Schwangerschaft und der Entstehung von Krebserkrankungen lassen sich verschiedene Parallelen ziehen. Im Rahmen der Implantationsphase des Embryos proliferieren und invadieren Trophoblastzellen ins mütterliche Gewebe, während eine komplexe Interaktion mit dem mütterlichen Immunsystem eine Immuntoleranz gewährleistet. In ähnlicher Weise sind viele Tumoren in der Lage, invasiv zu wachsen und sich vor dem Immunsystem zu maskieren. So scheinen sich die grundlegenden Mechanismen, die Embryoimplantation und Tumorprogression regulieren, zu ähneln. Der Janus Kinase / Signal Transducer and Activator of Transduction (JAK/STAT)-Signalweg spielt eine Rolle in der Transduktion einer Vielzahl von Signalen, die Zelldifferenzierung, Proliferation, Migration und Apoptose regulieren. Leukemia Inhibitory Factor (LIF) ist ein Zytokin mit maximaler Expressionsrate während der Implantationsphase und signalisiert über den JAK/STAT-Signalweg. Obwohl die Funktion von LIF noch nicht endgültig geklärt ist, wird vermutet, dass es durch die Aktivierung von STAT3 die Embryoimplantation unterstützt und Tumorinvasion verstärkt. Die Intention dieser Arbeit war, die Rolle von LIF sowie die des JAK/STAT-Signalweges in Chorionkarzinom-Zelllinien näher zu analysieren, die häufig als Modelle für Studien der Physiologie von Trophoblasten genutzt werden.

Methoden

Verschiedene Chorionkarzinom-Zelllinien wurden mit LIF und IL-6 stimuliert, um die Wirkung dieser Zytokine auf die Tyrosinphosphorylierung von STAT3 zu untersuchen. Nachgewiesen wurde dies mittels Western blot. Da die Übertragung des LIF-Signals vom Rezeptor auf die STAT-Moleküle über die Phosphorylierung von drei verschiedenen Januskinasen vermittelt werden kann, wurden diese ebenfalls mittels Western blot untersucht. Anschließend wurde Immunzytochemie durchgeführt, um die Ergebnisse zu bestätigen. Weiterhin wurde diese Methode angewendet, um die natürliche Expression der drei Januskinasen im Plazentagewebe einer gesunden Frau mit elektivem Abort zu untersuchen. Nachdem ermittelt wurde, welche Januskinasen nach LIF-Stimulation in den Chorionkarzinom-Zelllinien exprimiert und phosphoryliert werden, folgten Experimente zum Etablieren eines spezifischen Knockdowns der Kinaseexpression. Hierfür wurden verschiedene Transfektionsreagenzien und siRNA-Konzentrationen für die RNA-Interferenz von JAK1 getestet. Die Effizienz des Knockdowns wurde mittels Western blot evaluiert. Im Anschluss an diese Experimente folgten funktionelle Tests, um zu untersuchen, ob eine Behandlung mit LIF und/oder ein Knockdown der Kinase, Einfluss auf das Zellverhalten haben. Mithilfe der Durchflusszytometrie wurden Proliferations-, Migrations- und Invasionsraten der Zellen quantifiziert.

Ergebnisse

Wie im Western blot demonstriert wurde, korrelierte die Stimulation mit LIF mit der Phosphorylierung von STAT3 in Chorionkarzinom-Zelllinien. Die Stimulation mit IL-6 hingegen blieb ohne Effekt. Die Zellen zeigten hinsichtlich der natürlichen Expression und Phosphorylierung von Januskinasen Unterschiede. Eine natürliche Expression von JAK1 und JAK2 wurde in JAR-Zellen nachgewiesen, und die Stimulation mit LIF induzierte eine Phosphorylierung von JAK1. In JEG-3 Zellen sahen wir nur eine geringe Expression von JAK2, und die phosphorylierte Form zeigte nach LIF-Stimulation keinerlei Veränderung. Natürliches oder aktiviertes JAK3 konnten in keiner der beiden Zelllinien nachgewiesen werden. Die Immunzytochemie zeigte ähnliche Ergebnisse hinsichtlich der natürlichen Expression der JAK-Isoformen in diesen Zellen. JAK1 sowie JAK2, nicht aber JAK3, wurden in Plazentagewebe, hauptsächlich in villösen Trophoblasten, nachgewiesen. JAK1 wurde für die RNA-Interferenz gewählt, da es eine natürliche Expression in den JAR-Zellen zeigte, sowie nach LIF-Stimulation in phosphorylierter Form vorlag. Maximale Knockdown-Raten (ca. 65% JAK1 Knockdown) wurden mit Oligofectamin als Transfektionsreagens erzielt. In funktionellen Tests korrelierte die LIF-Stimulation mit einem signifikanten Anstieg der Proliferationsrate der JAR-Zellen. Der Knockdown von JAK1 induzierte eine Abnahme im Migrationsverhalten der Zellen.

Diskussion

Die Ergebnisse dieser Arbeit sollen für ein besseres Verständnis der Faktoren beitragen, die eine Rolle bei der Zytokinregulierung im JAK/STAT-Signalweg in Chorionkarzinomzellen spielen. Dieser Signalweg repräsentiert den Hauptsignalmechanismus für eine breite Gruppe von Wachstumsfaktoren und Zytokinen in Tieren. Die natürliche Expression der drei Januskinasen wurde in ähnlicher Form in verschiedenen Chorionkarzinom-Zelllinien als auch in Plazentagewebe gefunden. Weiterhin wurde untersucht, wie diese Isoformen durch LIF beeinflusst werden. Das Zytokin LIF wird als unentbehrlich während der Plazentationsphase in verschiedenen Spezies betrachtet und ist in die Regulierung von Tumorprogression involviert. Es wurde gezeigt, dass LIF eine STAT3-Aktivierung in allen untersuchten Chorionkarzinom-Zelllinien induziert. STAT3 spielt eine Rolle bei Zellbeweglichkeit, invasivem Wachstum und steht auch in Zusammenhang mit neoplastischem Zellverhalten. Dahingehend konnte demonstriert werden, dass LIF die Proliferationsrate von JAR-Zellen erhöht. Eine reduzierte JAK1-Expression durch RNA-Interferenz korrelierte mit verändertem Zellverhalten. Somit könnte der JAK/STAT-Signalweg als ein mögliches Ziel für molekül-basierte Therapien fungieren. Die Erforschung der Vielzahl von Faktoren und Signalwege, die Zellproliferation und –invasion regulieren, könnte zur Entwicklung neuer Therapien sowohl bei Krebserkrankungen als auch bei Schwangerschafts-assoziierten Erkrankungen beitragen.

1. Introduction

Pregnancy is a natural event that constitutes an enormous challenge to the maternal organism. The maternal immune system faces, during pregnancy, a situation in which it must tolerate and support the growth and development of the semi-allogeneic invading embryo. At the same time it combats potential pathogens that might impair gestational progression. In the course of embryo implantation, trophoblast cells are able to proliferate, migrate and invade into maternal tissues, while a complex interaction with the maternal immune system ensures the necessary immune tolerance. Interestingly, several parallels may be drawn between pregnancy and malignancies. Many tumors, resembling trophoblast cells, are able to grow invasively and to mask themselves from the immune system. The underlying mechanisms governing embryo implantation and tumor progression seem to lie in close proximity. The clarification of the multitude of factors and signaling pathways regulating cell invasion, both in the physiological and pathological settings, may lead to the development of new therapies targeting pregnancy related pathologies and cancer.

1.1 – Blastocyst Implantation and Trophoblast Invasion

Implantation of the human blastocyst into the maternal endometrium occurs at the 6^{th} or 7^{th} day after conception. For successful implantation, the blastocyst must firstly adhere to the luminal epithelium of a receptive endometrium (Herrler et al. 2003). The period of endometrial receptivity is known as “window of implantation” and is limited to the mid-secretory phase of the menstrual cycle (about days 19-23 of a standard 28-day cycle) (Psychoyos 1973). Important modifications of the endometrium elicited by estrogen and progesterone have occurred up to this phase and constitute a prerequisite to a successful implantation. The set of endometrial modifications culminates in the formation of the decidua (in a process generally referred to as decidualization). Among others, these modifications include (Dimitriadis et al. 2010):

- altered expression of extracellular matrix and cell surface molecules in the endometrial luminal epithelium,
- increased secretion by endometrial glands,
- differentiation of the periarteriolar fibroblasts in the stroma,
- influx of maternal immune cells (mainly natural killer cells) into the uterus.

Upon attachment to the receptive endometrium, blastocyst cells proliferate and start to migrate across the endometrial luminal epithelium, infiltrating into the maternal tissues. The so-called trophoblast derivates from the external layer of the blastocyst (called trophectoderm) and constitutes the extra-embryonic tissue that is directly involved in the processes of proliferation, migration and invasion, finally forming the placenta. The trophoblast comprises different populations of cells, such as the actively proliferating cytotrophoblast, also referred to as trophoblast stem cells, from which further differentiation will originate different cell subsets (Ferretti et al. 2007).

The process of blastocyst adhesion to the endometrial epithelium is believed to trigger the differentiation of the cytotrophoblast into two distinct subsets, the villous and extravillous populations (Fig. 01) (Armant 2005). In the villous pathway, the villous cytotrophoblast remains in the fetal compartment and fuses to form the syncytiotrophoblast, a multinucleated structure with endocrinal activity into which the blastocyst embeds (Bischof and Campana 1997). Since it covers the floating chorionic villi and the intervillous space, the syncytiotrophoblast is in direct contact with the maternal blood and thus participates in the fetal-maternal exchange. In the extravillous pathway, the extravillous cytotrophoblast (EVT) forms the stratified cylinder-shaped structure called cell column, whose distal part differentiates into an invasive phenotype (Kaufmann and Castellucci 1997). A subpopulation of the EVT, called interstitial EVT, infiltrates into the uterine decidua and anchors chorionic villi into the Nitabuch layer (the fibrinoid layer between the compact endometrium and the cytotrophoblastic shell). Another subpopulation, known as endovascular EVT, invades into a third of the depth of the spiral arteries in the myometrium and engrafts the maternal blood vessels, creating low resistance vessels that will provide adequate perfusion (assuring oxygen and nutrients) to the forming fetus (Genbacev et al. 1992, Dimitriadis et al. 2010).

Meanwhile, during the process of embryo implantation, the decidua (including the decidualized stromal cells and maternal leukocytes) continues to develop and forms the uterine line that will constitute the maternal part of the placenta, where maternal cells are directly in contact with fetal cells (Veenstra van Nieuwenhoven et al. 2003). The decidua has been implicated in the regulation of trophoblast invasion and plays thus an essential role in pregnancy (Dimitriadis et al. 2010). The observation that uncontrolled trophoblast invasion occurs in sites where the decidua is partially or totally absent (as observed in ectopic pregnancies) supports this regulatory property of the decidua (Lockwood et al. 2008). Moreover, a correlation between human infertility and ineffective decidualization of

endometrial stromal cells has been identified *in vitro* (Karpovich et al. 2005), suggesting that the successful establishment of pregnancy depends on adequate decidualization.

Finally, the process of trophoblast invasion, which had started in the early stages of embryo implantation, forms a continuum which extends during the whole pregnancy. Up to the 8th week of pregnancy, the uterine mucosa has been extensively colonized by interstitial EVT, which become rounded and multinucleated (forming the placental giant cells) (Ferretti et al. 2007). These cells continue to invade the inner myometrium during the second and third trimester, although in a much more decreased intensity. In these phases, most of the interstitial EVT cells are morphologically giant cells.

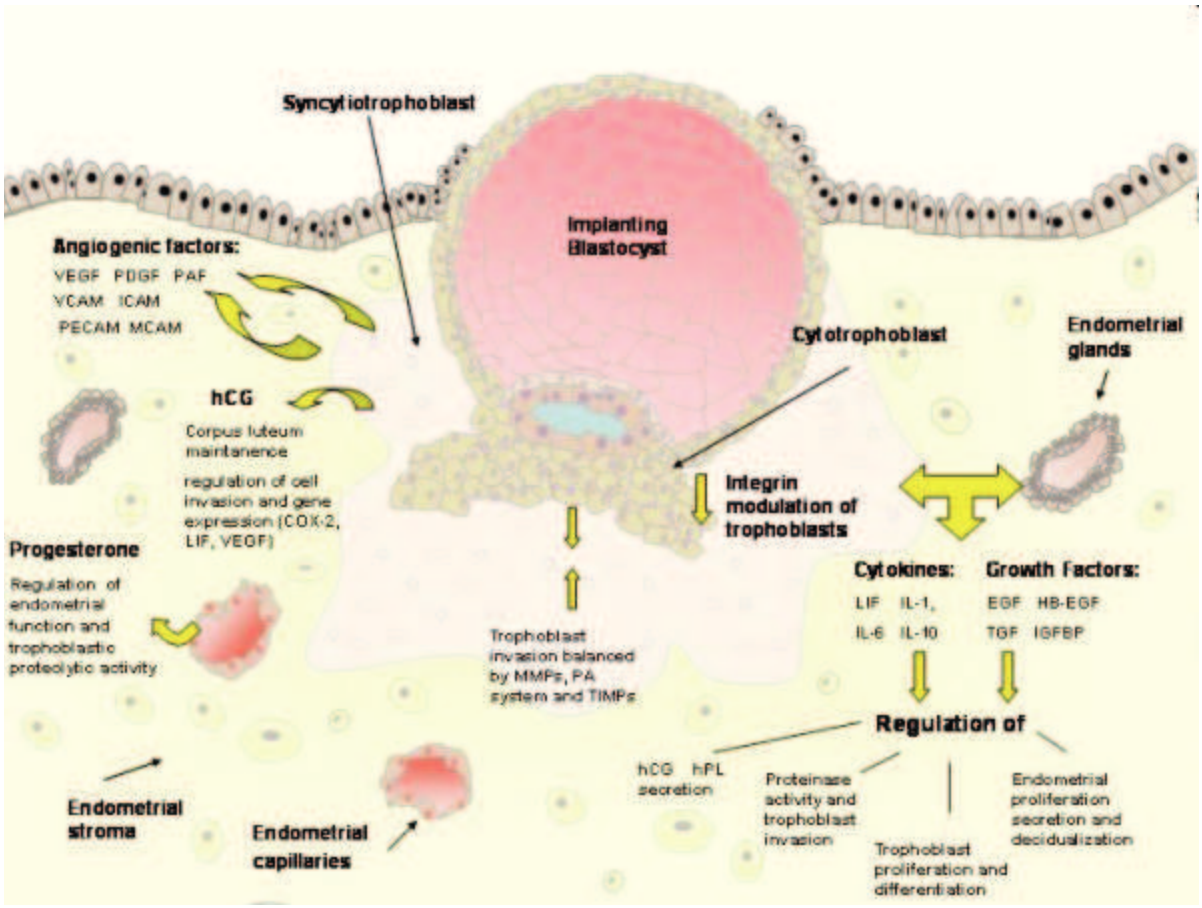


Fig. 01 Implanting blastocyst (Figure from Staun-Ram and Shalev 2005)

Blastocyst implantation constitutes a complex process involving a multitude of factors regulating cell proliferation, migration and invasion. These factors are believed to be controlled by the blastocyst itself and the maternal microenvironment. The figure above highlights the interaction between the trophoblast and maternal endometrium, including cytokines, growth factors, integrins, proteases and hormones (Staun-Ram and Shalev 2005).

1.2 – The Immune System During Pregnancy

For many years the fetal-maternal boundary had been believed to be an immunologically inert site, in which any contact between fetal antigens and maternal immune system should be avoided. The discovery of anti-paternal, anti-fetal and anti-placentary antibodies in the serum of pregnant women (Billington 1992) changed this premise, suggesting that the maternal immune system recognizes and reacts to fetal-derived antigens in a very controlled manner, in which embryo development is not disturbed. It is now accepted that recognition of fetal antigens by the maternal immune system is a prerequisite to a successful pregnancy outcome. This recognition leads to appropriate immunological tolerance, enabling the embryo to growth and develop, while harmful pathogens are combated (Szekeres-Bartho 2002, Schmitt et al. 2008).

Considering that about half of the antigens expressed by the fetus derives from the father (therefore potential targets of immune attack), it is not difficult to imagine that pregnancy represents a big challenge to the maternal immune system, which needs to recognize the embryo, a "semi-allograft organism", without being harmful to it, at the same time that it needs to confront undesired pathogens. In these terms, several mechanisms have been shown to participate in the puzzle of immune tolerance, such as the expression of Major Histocompatibility Complex (MHC) molecules by the trophoblast and important changes in the balance between T-helper 1 (Th1) and T-helper 2 (Th2) cytokines.

The trophoblast lack many of the classical MHC molecules (such as MHC classes Ia and II), but expresses a special non-polymorphic non-classical MHC Ib molecule – the Human Leukocyte Antigen G (HLA-G), which can be recognized by different decidual leukocyte populations (Exley and Boyson 2011). The non-expression of classical MHC molecules leads the trophoblast to express just a few paternal antigens – a mechanism used to escape from T-cell-mediated lyses (Geis and Dietl 2001). Furthermore, HLA-G has been implicated in inhibition of the cytolytic activity of NK-cells, rescuing the embryo from a potentially harmful immune attack (King et al. 2000). HLA-G is mainly expressed by extravillous trophoblast cells, which keep direct contact with maternal immune cells in the decidua.

The process of antigen identification is mediated by maternal γδT-cells, which are able to recognize molecules in a MHC-unrestricted manner and without antigen processing or presentation (Mincheva-Nilsson 2003). In the decidua of early pregnancy, more than half of the γδT-cells express PR, which is considered a marker for their activity. Active γδT-cells have been demonstrated to modulate maternal immune system towards immune tolerance.

They also act against undesired pathogens and regulate trophoblast invasion (Poehlmann et al. 2006).

Cytokines and Th1/Th2 Balance in Pregnancy

Cytokines encompass a large family of signaling molecules, including proteins, peptides and glycoproteins. They play an essential role in several processes of intercellular communication, such as immune modulation (Gilman et al. 2001). For a successful implantation and fetal-placental development, it is necessary to highlight the role of Th1 and Th2 cytokines during pregnancy.

From the beginning of implantation up to term, important modifications are observed in the balance between these cytokines, and these changes are considered indispensable for a successful pregnancy outcome (Dealtry et al. 2000). Th1 cytokines, such as TNF, IL-2, IL-12 and IFN-γ, induce inflammatory reaction and cytotoxicity upon activation of macrophages. They seem to be particularly important in the peri-implantation period, when they induce a local proinflammatory environment that has been suggested to facilitate initial trophoblast invasion (Joachim et al. 2003). Subsequently, an important shift towards Th2 dominance is observed as placentation begins, and this dominance is maintained up to the end of pregnancy (Dealtry et al. 2000). Th2 cytokines, such as IL-4, IL-6, IL-10, IL-13 and TFG-β, induce humoral immunity mediated by mast cells and eosinophils, and create an anti-inflammatory environment in the fetal-maternal interface that has been considered indispensable for successful placentation and fetal survival (Veenstra van Nieuwenhoven et al. 2003).

In fact, disturbances in the balance of Th1/Th2 cytokines and the undesired dominance of Th1 cytokines throughout pregnancy have been associated with impairment of trophoblast function and embryo development, culminating in recurrent abortion (Geis and Dietl 2001, Joachim et al. 2003). Furthermore, the physiological dominance of Th2 cytokines during pregnancy (and its consequent anti-inflammatory role) is recognizable by the fact that women suffering from autoimmune disorders (such as rheumatoid arthritis and collagenosis) usually encounter alleviation of their symptoms during pregnancy. On the other hand, the reduced cell-mediated immunity triggered by Th2 cytokines also determines an undesired increase in the incidence of viral infections and malignancies in pregnant women (Geis and Dietl 2001, Szekeres-Bartho et al. 2001). Finally, at the end of pregnancy, a new shift towards Th1 dominance is achieved, and this restored pro-inflammatory environment is believed to play a role in the process of birth and in maternal and child immune defense (Dealtry et al. 2000).

1.3 – Parallels between Pregnancy and Cancer

Blastocyst implantation and subsequent placentation depend on the capability of the newly formed extravillous trophoblast to invade into the uterine decidua and to engraft maternal blood vessels (Dimitriadis et al. 2010). This continuous process demands an appropriate and complex cross-talk between the mother and the forming fetus, in which several molecules participate, including growth factors, cytokines, chemokines, proteases and adhesion molecules (Salamonsen et al. 2009).

Trophoblast invasion is a complex process that must be spatially and temporally regulated to ensure the proper development of a functional placenta and the establishment of pregnancy (Dimitriadis et al. 2010). Taken its proliferative and invasive properties, and considering the fact that the embryo possesses paternal antigens that might trigger maternal immunological reaction, the forming fetus can be ultimately considered a semi-allogeneic tissue, which is able to invade and induce host-immunotolerance (Murray and Lessey 1999). Interestingly, many malignancies mimic and abuse of these physiological mechanisms of cell invasion and immunological masking in disadvantage to the "host" (Fitzgerald et al. 2008). The highly proliferative phenotype of EVT cells closely resembles the tumorigenic phenotype of localized tumors after the neoplastic cell transformation (Gupta et al. 2005). In these terms, both trophoblast and tumor cells share an intense proliferative behavior and display a lack of cell-cell contact inhibition. Furthermore, the migratory and invasive phenotype of EVT cells is similar to the metastatic phenotype of tumor cells subsequent to their malignant transformation. Both cells subsets share here the aptitude to attach to the basement membrane and to promote it proteolysis, in an active process of membrane penetration (Poste and Fidler 1980, Staff 2001). But differently from tumor cells, the physiological trophoblast invasion occurs in a tightly controlled manner (Bischof and Campana 2000, Kliman 1993). This is supported by the fact that trophoblast cells from term placenta possess a decreased ability to invade (Poehlmann et al. 2005).

The similar mechanisms governing embryo implantation and tumor development, including their underlying cell signaling pathways, make the study of invasion an important task to be accomplished. The knowledge about the extra and intra-cellular signals that regulate invasion remains meanwhile elusive, and the understanding of this complex process might thus contribute to elucidate tumor progression and guide the development of new approaches targeting cancer and pregnancy-related diseases.

Choriocarcinoma, a Gestational Trophoblastic Neoplasia

Gestational trophoblastic disease (GTD) embraces a spectrum of benign and malignant tumors derived from the placental villous trophoblast (Lurain 2010). GTD is usually subdivided in:

- hydatidiform mole (complete and partial), a benign but potentially deadly tumor;
- gestational trophoblastic neoplasia (GTN), which encompasses invasive mole, choriocarcinoma and placental site trophoblastic tumor.

GTN is composed of malignant tumors, which can invade and metastasize, leading invariably to death when untreated (Soper 2006, Berkowitz and Goldstein 2009). Among them, choriocarcinoma is a rare condition affecting about 1 in 40,000 pregnancies in Europe and North America (Brinton et al. 1986, Smith et al. 2003). It is characterized by the lack of chorionic villi, abnormal trophoblastic hyperplasia and anaplasia, necrosis and hemorrhage. Malignant cells are able to invade into myometrium and its blood vessels, commonly metastasizing to the lungs, liver, brain, pelvis and vagina. Choriocarcinoma arises in 50% of the cases from hydatidiform moles, but it can be associated with any pregnancy event, such as tubal pregnancy, preterm or term gestation (Lurain 1990). In fact, prior complete hydatidiform mole constitutes the principal risk factor for the development of choriocarcinoma, although less than 4% of moles progress to it (Lurain 1990). Advanced maternal age and ethnicity constitute further risk factors (the condition is more common in Asian descents and African Americans) (Palmer 1994, Smith et al. 2003).

Clinically, postmolar choriocarcinoma usually presents as irregular bleeding subsequent to mole evacuation, in conjunction with persistent enlargement of the ovaries, uterine subinvolution and irregularity. hCG levels persist abnormally plateaued or get elevated after mole evacuation. Signs of non-postmolar choriocarcinoma are unspecific and commonly arise as the tumor invades the uterus or metastasizes to distant organs, leading to pulmonary, gastrointestinal and/or neurological symptoms. In these cases, elevated hCG levels are usually identified along with the discovery of metastasis (Lurain 2010). Pathologically, the diagnosis of choriocarcinoma can be made by curettage, evaluation of placental or hysterectomy specimens and by the biopsy of metastasis.

Chemotherapy constitutes the standard treatment for GTN (Smith et al. 2005). Depending on staging, single or multiple-agent chemotherapy is chosen. Methotrexate or actinomycin D is usually administered in patients with nonmetastatic or low-risk GTN (Foulmann et al. 2006, Roberts and Lurain 1996, Osborne et al. 2011). Patients with high-risk metastatic GTN,

particularly those at high risk of therapy failure, are usually treated with a combination of drugs (Lurain et al. 2006, Escobar et al. 2003, Xiang et al. 2004). Response to treatment is evaluated by serum hCG levels every week. Nowadays, GTN constitute some of the most curable solid tumors, even in the presence of metastasis (Lurain 2010).

1.4 – The JAK/STAT Signaling Pathway

The Janus Kinase / Signal Transducers and Activators of Transcription (JAK/STAT) pathway is one of the main signaling mechanisms in mammals, used to transduce the signals of a broad spectrum of cytokines and growth factors (Rawlings et al. 2004). This pathway has been implicated in the transduction of a multitude of signals governing development and homeostasis, including cell proliferation, differentiation, migration and apoptosis (Bromberg 2001). These events are essential for several physiological processes, such as immune regulation, hematopoiesis, adipogenesis, mammary gland development etc (Rawlings et al. 2004). Predictably, dysfunction of the JAK signaling (aberrant activation or impaired regulation) has been associated with pathological conditions, such as erythrocytosis, inflammatory diseases and leukemias (Rawlings et al. 2004, Igaz et al. 2001).

JAK/STAT activation occurs upon binding of specific ligands on cell surface, inducing multimerization of receptor subunits (which are bound as homo or heterodimers), whose cytoplasmic domains are associated with two tyrosine kinases - the Janus kinases (JAKs). The JAKs structure reveals two similar phosphate-transferring domains at it C-terminus, one with kinase activity and the other one with negative regulatory property. By the way, the name Janus Kinases derives from this “double-headed” structure, resembling the two-headed Roman mythological god Janus (Fitzgerald et al. 2010).

After ligand binding promotes receptor multimerization and juxtaposition of the two receptor-associated JAKs, these are brought into close proximity and activate each other through cross-phosphorylation. Furthermore, the activated JAKs promote phosphorylation of additional targets, including inner substrates and specific cytoplasmic domains of the JAK-associated receptor (Fitzgerald et al. 2008). Among them, intracellular STATs are latent transcriptional factors residing in the cytoplasm and constitute a major substrate for JAKs phosphorylation. After binding to the phosphorylated receptor domains, the latent STATs in the vicinity of the activated JAKs are then phosphorylated by the latter and dissociate to form homo and heterodimers, which translocate to cell nucleus by the importin α-5 (also known as

nucleoprotein interactor 1) and the Ran nuclear import pathway. In the nucleus, these homo and heterodimers bind and manipulate the promoter regions of target genes, ultimately influencing their transcription (Rawlings et al. 2004).

Furthermore, the JAK/STAT signaling pathway is modulated by negative regulators, such as the Protein Tyrosine Phosphatases (PTPs), the Suppressors of Cytokine Signaling (SOCS) and the Protein Inhibitors of Activated STATs (PIAS). Direct and indirect mechanisms lead to negative modulation of the JAK/STAT cascade. For example, some PTPs (such as the SHP-1) bind to phosphorylated JAKs and receptor domains, facilitating their dephosphorylation (Greenhalgh and Hilton 2001). Moreover, the SOCS negatively modulate the JAK/STAT pathway by different means, such as by inhibition of the JAK kinase activity upon direct binding to JAKs and JAKs-associated receptors. Some SOCS also can bind phosphotyrosines on receptor domains, what physically blocks them and prevents STATs recruitment. Some SOCS also facilitate ubiquitination of JAKs through interaction with the elongin BC complex and cullin 2, ultimately targeting the JAKs for proteasomal degradation (Alexander 2002). Interestingly, the expression of SOCS proteins is stimulated by the activated STATs, revealing a negative feed-back loop (Naka et al. 1997). Finally, the PIAS proteins bind to phosphorylated STAT dimers, preventing them to bind DNA (Rawlings et al. 2004).

1.5 – Interleukin-6 (IL-6)

Active IL-6 is a 184 amino acid-long cytokine, which is able to exert both pro- and anti-inflammatory responses (Singh et al. 2011). Among others, it is synthesized by monocytes, macrophages, fibroblasts, T cells and endothelial cells. It is one of the most important mediators of the acute phase response, as well as an effector of the humoral immune response, inducing the syntheses of immunoglobulins. Besides inducing proliferation and differentiation of B- and T-cells, IL-6 is also involved in hematopoiesis, fever, osteoclast development, ACTH production etc (Aguzzi and Heikenwalder 2005, Moser and Willimann 2004, Ohsuzu 2004, Gosain and Gamelli 2005, Heinrich et al. 1998).

IL-6 activity is mediated by the binding of this cytokine to its type I cytokine receptor complex on cell surface, which is composed of the ligand-binding IL-6Rα chain and the 130 kDa signal transducer protein (gp130) (Hibi et al. 1990, Yamasaki et al. 1988). The interaction leads to multimerization of these receptor subunits, activating the receptor. The activation promotes intracellular juxtaposition of the two receptor-associated JAKs, which are

brought into proximity and phosphorylate each other. IL-6 thus signals through the JAK/STAT pathway (Heinrich et al. 1998). IL-6 receptor is present on maternal and fetal tissues at the time of implantation, both in humans and in mice, suggesting a paracrine/autocrine role (Kojima et al. 1995, Tabibzadeh et al. 1995).

IL-6 is mostly expressed in the endometrium (mainly in the epithelial glandular cells) in mid- to late-secretory phase (Singh et al. 2011, Tabibzadeh et al. 1995). The period of maximal expression coincides with the time of implantation window, inviting the hypotheses that this cytokine might play a role in the process of embryo invasion and implantation in early pregnancy. In fact, IL-6 has been demonstrated to induce proliferation and migration of trophoblast cells *in vitro* (Fitzgerald et al. 2005a), as well as to enhance the activity of metalloproteinases 2 and 9 (MMP-2 and MMP-9), which are involved in the degradation of extracellular matrix, thus enabling the trophoblast to invade the endometrium and reach maternal blood supply (Meisser et al. 1999). Furthermore, dysregulation of IL-6 expression seems to be associated with impairment of pregnancy. In these terms, IL-6 levels have been reported to be reduced during the mid-secretory phase in patients suffering from recurrent spontaneous abortion (von Wolff et al. 2000). In murine models, IL-6 deficiency correlates with reduced implantation sites and reduced fertility (Singh et al. 2011). In these models, even when implantation is achieved, blastocysts are found to be underdeveloped (Salamonsen et al. 2000, Kopf et al. 1994).

Besides its roles during early pregnancy, IL-6 has also been linked to cancer progression. This cytokine and the members of its family are usually observed in tumor microenvironments, where they promote the attraction of inflammatory cells, leading to an increased expression of proteases and proinvasive proteins (Sanz-Moreno et al. 2011, Coussens and Werb 2002). Additionally, IL-6 has been linked to metastatic progression in some malignancies, including ovarian, head and neck cancer, in which elevated levels of this cytokine in the serum have been associated to increased cell invasiveness (Nishino et al. 1998, Obata et al. 1997). In melanoma and colorectal cancer, IL-6 levels have been found extra-elevated in patients with advanced pathological stage, and preoperative measurement of serum IL-6 has thus been suggested as a potential biomarker of tumor metastases (Eldesoky et al. 2011).

1.6 – Leukemia Inhibitory Factor (LIF)

Leukemia Inhibitory Factor (LIF) is a multifunctional 179 amino acid-long interleukin class 6 cytokine initially discovered in the conditioned medium of rat liver cells (Omori et al. 1996). Its name comes from the ability of this molecule to induce the terminal differentiation of M1 myeloid leukemic cells into macrophages (Tomida et al. 1984).

This cytokine is expressed in different cell types, including neurons, ovarian stromal cells, breast, hepatocytes and kidney epithelial cells (Lass et al. 2001, Auernhammer and Melmed 2000). Considered a Th2-type cytokine, LIF key functions point to mediation of inflammatory cell responses (Okahisa et al. 2010, Knight et al. 1999). Since its discovery, LIF has been proposed to be a multifunctional cytokine, playing a role in several physiological and pathological processes, including placentation in several species, trophoblast and human choriocarcinoma invasion, neural development, bone metabolism, inflammation, cancer cachexia, protection of photoreceptors from light damage etc (Burgi et al. 2009, Sims and Johnson 2012, Simamura et al. 2010, Kamohara et al. 2007, Fitzgerald et al. 2005b).

On the cell surface, LIF binds to its specific low affinity receptor subunit (LIFR), triggering dimerization with it heterodimer, the transmembrane glycoprotein 130 (gp130), which is a STAT3-activating subunit common to all members of IL-6 receptor family. The binding forms a high affinity receptor and promotes juxtaposition of the receptor-associated Janus Kinases (JAKs), which reciprocally activate each other through cross-phosphorylation. In these terms, IL-6 type cytokines (among them LIF) have been reported to activate JAK1, JAK2 and TYK2 (Heinrich et al. 1998). The activation of JAKs leads in turn to phosphorylation of the cytokine receptors and of inner ligands, such as STAT3. Activated STAT3 then dissociate to form homo and heterodimers and translocate to cell nucleus, where they influence the transcription of target genes (Yamaoka et al. 2004). These events are summarized in Fig. 02.

Finally, LIF signaling response is negatively modulated by the Suppressors of Cytokine Signaling (SOCS), whose action mechanism was previously described. Interestingly, SOCS3 constitutes a STAT-induced protein which expression is stimulated by the LIF/JAK/STAT3 pathway. In these terms, LIF suppresses its own effect through a negative feedback (Fitzgerald et al. 2008, Naka et al. 1997).

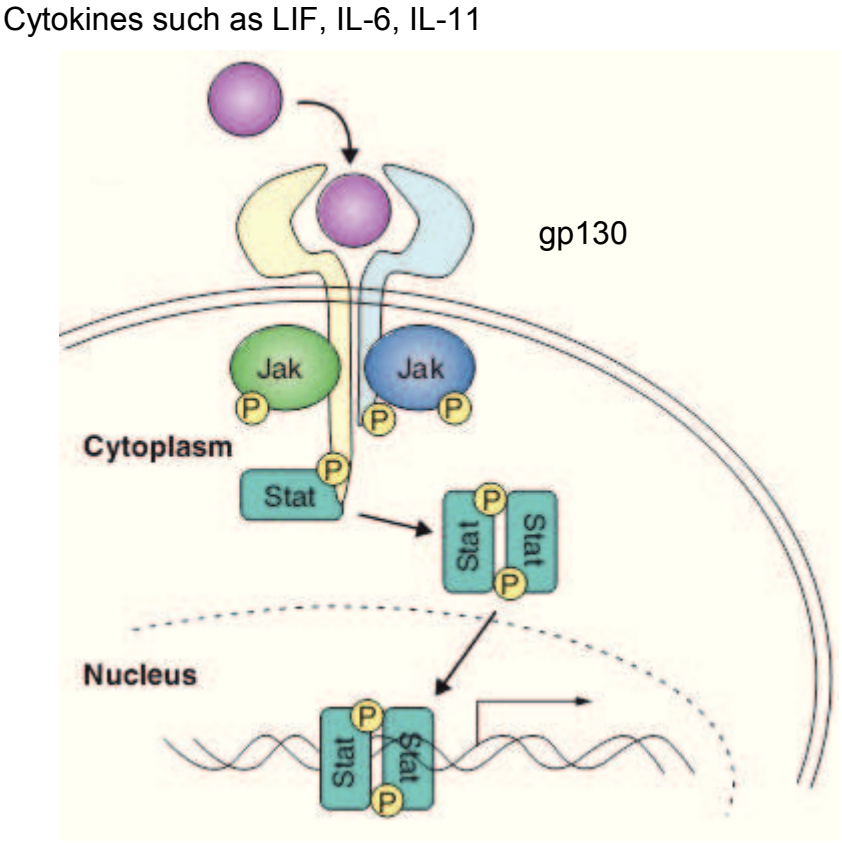


Fig. 02 JAK/STAT signal transduction pathway (Modified from Yamaoka, 2004)
Cytokines of the IL-6 family bind to gp130 receptors, which are constitutively bound to two JAKs, leading to JAK cross-phosphorylation, what in turn leads to phosphorylation of the cytokine receptors. STAT proteins in the vicinity of these receptors are phosphorylated by activated JAKs, promoting STAT dimerization. STAT dimers translocate to cell nucleus, where they induce gene expression.

LIF and Pregnancy

LIF is assumed to facilitate embryo implantation and is considered indispensable during placentation (Paiva et al. 2009). Produced by human placenta and endometrium (especially in the glandular and luminal epithelium and in the endometrial stroma), its expression is maximally observed in the maternal-fetal interface at the time of implantation (Vogiagis et al. 1996, Aghajanova et al. 2003, Bhatt et al. 1991). Furthermore, LIF mRNA is intensively expressed in decidual leukocytes present at the implantation site, suggesting a cross-talk between maternal leukocytes and the invading trophoblast via LIF (Sharkey et al. 1999).

As blastocyst approximates to the endometrium, the latter expresses LIF at high levels, while the former produces LIF receptor, revealing a paracrine signaling (Cullinan et al. 1996, Tapia et al. 2008). LIF has been demonstrated to enhance extravillous trophoblast adherence to extracellular matrix components, such as fibronectin, vitronectin and laminin *in vitro* (Tapia et al. 2008). Furthermore, LIF increases the adhesion of human endometrial epithelial cells to collagen IV, present on the surface of blastocysts and first trimester trophoblast cells (Marwood et al. 2009, Shimomura et al. 2006, Kurosawa et al. 1985). After adhering to the endometrium, the blastocyst commences to express LIF mRNA itself, while LIF receptor is then expressed in the endometrium (Chen et al. 1999, Charnock-Jones et al. 1994). Additionally, an extra production of endometrial LIF is stimulated upon blastocyst adhesion, especially after the newly formed syncytiotrophoblast invades into the luminal epithelium and starts to express LIF-stimulating cytokines (such as IL-1) (Aghajanova 2004, Laird et al. 2000).

The LIF-induced changes in the adhesive properties of the endometrial epithelium have been considered essential for blastocyst attachment and constitutes a first step for subsequent cell migration and anchorage of the trophoblast in the maternal decidua (Dimitriadis et al. 2010). Over the last years it has been investigated which factors present in the maternal-fetal interface might regulate the expression of adhesion molecules, such as integrins and cadherins. Adhesion molecules are important for the interaction between cells and extracellular matrix and are thought to modulate trophoblast migration and invasion (Irving and Lala 1995, Burrows et al. 1996). For example, the cognate $\alpha_5\beta_1$ integrin receptor present on the cell surface of extravillous trophoblast has been demonstrated to interact with fibronectin. This interaction influences the migratory activity of trophoblast cells, particularly where anchoring villi are formed (Ilic et al. 2001).

In this context, and considering its maximal expression at the time of implantation, LIF has been investigated as a potential factor regulating the expression of adhesion molecules. However, its role in the modulation of integrins has found some controversy. As aforementioned, LIF has been demonstrated to increase the adhesion of extravillous trophoblast cells to fibronectin, vitronectin and laminin *in vitro*, particularly to the last (Tapia et al. 2008). LIF had been thought to modulate the expression of their respective integrin receptors on cell surface (such as $\alpha_5\beta_1$ integrin receptor for fibronectin and $\alpha_6\beta_4$ integrin receptor for laminin), but, interestingly, no influence on the expression of the integrin subunits α_1, α_5 and β_1 has been demonstrated in trophoblast cells treated with LIF (using fluorescence-activated cell sorter analysis) (Sharkey et al. 1999). Moreover, LIF treatment has

been associated to a decreased mRNA expression of the β_4 subunit in trophoblast cells, as verified by real-time RT-PCR analysis (Tapia et al. 2008). Taken together, these data suggest that the increased adhesion of trophoblast to these extracellular matrix components might involve the regulation of other integrin subunits rather than α_1, α_5, β_1 and β_4. It is also plausible that LIF might act in concert with other factors to regulate trophoblast integrins (Luo et al. 2007). Especially the LIF-induced inhibition of the β_4 subunit has been focus of interest, since the differentiation of trophoblast cells of the cell column into an invasive phenotype has been linked to the downregulation of the integrin $\alpha_6\beta_4$ (Aplin 1997, Damsky et al. 1992). This inhibition ultimately supports the hypotheses that LIF triggers the process of trophoblast differentiation towards an invasive phenotype.

LIF has also been implicated in direct regulation of trophoblast invasion, since it may inhibit (Fitzgerald et al. 2005b) or stimulate the production of tissue inhibitors of metalloproteinases (TIMPs), which act as counterparts of matrix metalloproteinases (MMPs). MMPs are directly involved in the degradation of extracellular matrix, which is necessary to enable the trophoblast to invade the endometrium and reach maternal blood supply. Acting as counterpart, the TIMPs prevent excessive trophoblast invasion, ultimately contributing to a proper decidual invasion. This regulative effect supports previous studies demonstrating that LIF reduces gelatinase activity of trophoblast cells (Bischof et al. 1995) and suggests an overall inhibitory effect on trophoblast invasion.

Finally, it must be considered that the role of LIF and its underlying signaling pathway have not been completely clarified. As many other cytokines, its exact mechanisms and possible crosstalks with other signaling cascades are still partially understood (Fitzgerald et al. 2008).

Adequate trophoblast proliferation, migration and invasion are critical events for proper anchorage of the fetus to maternal endometrium and demand strict control of inter and intracellular signaling (Tapia et al. 2008, Genbacev et al. 1992). Dysregulation of the underlying mechanisms governing embryo implantation has been associated to adverse pregnancy outcome, leading in some cases to infertility (Dimitriadis et al. 2010). In this sense, LIF is considered critical for implantation, along with other cytokines, and disturbances in the fine regulation of its underlying JAK/STAT signaling pathway have been repeatedly associated to impairment of human and murine pregnancy (Dimitriadis and Menkhorst 2011). In this context, it is reasonable to think that studies investigating the role of LIF and its underlying signaling pathway might contribute to the better comprehension of the factors influencing embryo implantation and ultimately the factors necessary for a successful pregnancy outcome.

LIF, STAT3 and Cancer

Signal Transducer and Activator 3 (STAT3) is a signal-transduction mediator that is activated by several cytokines and growth factors. STAT3 signaling pathway has been demonstrated to participate in the development of pluripotent cells and tumors (Levy and Lee 2002), playing a role, among others, in cell motility and protease regulation (Fitzgerald et al. 2008, Zhang et al. 2002). Interestingly, dysregulation of STAT3 activity has been demonstrated to play a role in malignant cell transformation and tumor metastasis (Horiguchi et al. 2002). In these terms, aberrant STAT3 activity has been associated with the up-regulation of genes involved in cell cycle progression (e.g. c-myc) and apoptosis inhibition (e.g. mcl-1), posing the STAT3 protein as a candidate with oncogenic potential (Fitzgerald et al. 2010). The aberrant activity leads to malignant cell behavior, which embraces hyperplasia, longevity and invasiveness (Epling-Burnette et al. 2001, Bromberg et al. 1999). Indeed, constitutively phosphorylated STAT3 has been identified in different malignancies, including brain, breast, prostate and skin cancer, in contrast to its transient nature in normal cells (Niu et al. 2002, Schaefer et al. 2002, Dhir et al. 2002, Garcia et al. 2001).

The factors influencing/inducing the aberrant activity of STAT3 in malignant cells have not been completely clarified. Considering that LIF directly signals through the JAK/STAT pathway, this cytokine poses as a potential modulator of STAT3 activity in tumor cells. As a matter of fact, LIF has been demonstrated to induce STAT3 activation in JEG-3 cells, a human choriocarcinoma cell line used as model for invasive, first trimester trophoblast. In these cells, LIF has been proposed to induce malignant behavior, stimulating cell proliferation and invasion *in vitro* (Fitzgerald et al. 2005b). Furthermore, the LIF-driven STAT3 activity has been related to the down-regulation of TIMP-1 (Fitzgerald et al. 2005b). As it is known, TIMP-1 possesses an anti-invasive activity, since it inhibits all metalloproteinases, including latent and active MMP-9 (Lala et al. 2002, Goldberg et al. 1992). TIMP-1 has also been linked to inhibition of metastasis (Bischof et al. 2001). In this sense, the LIF-induced down-regulation of TIMP-1 has been suggested to induce the hyperinvasiveness of these cells (Fitzgerald et al. 2005b). On the other hand, LIF has also been assumed to induce an overall inhibitory effect on trophoblast invasion, as previously commented (Tapia et al. 2008, Bischof et al. 1995). Taken together, these data reveal that the influence of LIF on cell behavior has not been completely understood. Its role might even vary among cells from the pathological and physiological settings. In this sense, this work intended to better clarify the role of LIF

and the JAK/STAT signaling pathway in choriocarcinoma cell lines, which are considered models for studying trophoblast physiology.

2. Objectives

The goal of this work was to clarify some of the components of the JAK/STAT pathway that are involved in the signal transduction of LIF in choriocarcinoma cell lines. With the aid of functional assays, it has also been investigated the effects of this cytokine on cell behavior. Finally, the same functional parameters have been analyzed after performing JAK1 knock-down using the technique of RNA interference, in order to investigate if disturbances in the JAK/STAT signaling pathway might alter the behavior of these cells.

Steps

- Investigation of a possible correlation between LIF and IL-6 stimulation and STAT3 phosphorylation in different choriocarcinoma cell lines by applying Western blots
- Determination of which JAK isoforms are constitutively expressed in choriocarcinoma cells and placental tissues by applying Western blots and immunocytochemical studies
- Determination of which JAK isoforms are phosphorylated upon LIF stimulation in choriocarcinoma cells by applying Western blot
- Investigation of LIF effects on cell behavior by performing functional assays (proliferation, migration and invasion)
- Establishment of an efficient JAK1 knock-down using the technique of RNA interference. To achieve this goal, different transfection reagents and siRNA concentrations have been tested
- Evaluation of knock-down efficiency by means of Western blot
- Evaluation of STAT3 phosphorylation in JAK1-silenced cells using Western blot
- Investigation of the effects of JAK1 knock-down on cell behavior by performing functional assays (proliferation, migration and invasion)
- Investigation of the effects of LIF stimulation on the proliferative, migratory and invasive behavior of JAK1-silenced cells

Questionnaire

- Do LIF and IL-6 induce tyrosine STAT3 phosphorylation in choriocarcinoma cell lines?
- Are JAK1, JAK2 and JAK3 constitutively expressed in choriocarcinoma cells and placental tissues?

- Is LIF associated with the phosphorylation of these JAK isoforms in choriocarcinoma cells?
- Does the reduced expression of JAK1 influence the LIF-induced STAT3 phosphorylation in JAR cells?
- Does LIF stimulation influence the proliferative, migratory and invasive behavior of choriocarcinoma cells?
- Is the LIF-induced cell behavior altered in choriocarcinoma cells with reduced JAK1 expression?

3. Material and Methods

All experiments were performed in the Placenta Laboratory of the Department of Obstetrics, University Hospital Jena, under the supervision of Prof. Dr. med. Udo R. Markert. Many of the following methods were performed more than once. In this chapter, material and methods are briefly described in order to avoid unnecessary repetition and each method is correlated with its specific aim. Material origins and further specifications are presented in the last part of this work.

3.1 – Cell Lines

Human Choriocarcinoma Cell Lines JAR and JEG-3

Many experiments in this work were performed using JAR and JEG-3 cells (Fig. 03 and 04), which are human choriocarcinoma cell lines (hCC) derived from first trimester trophoblast (Pattillo et al. 1971). Originally, JAR cells have been established from a placental trophoblastic tumor of a 24-year-old Caucasian woman. JEG-3 cells have been isolated from a cerebral metastasis of a human gestational choriocarcinoma (Pattillo and Gey 1968, Kohler and Bridson 1971).

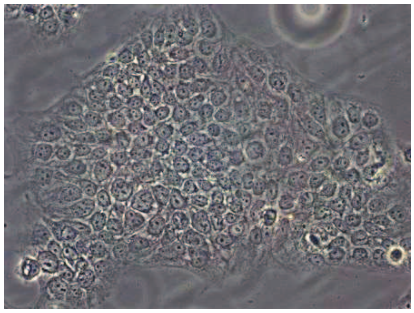


Fig. 03 JEG-3 cells

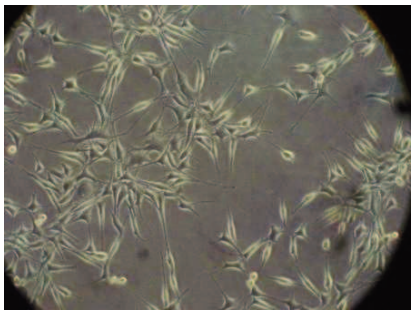


Fig. 04 JAR cells

JAR and JEG-3 cells have been chosen in this work because they are considered models for studying trophoblast physiology. These cells present several similarities with trophoblasts, such as the production of pregnancy related hormones, including ß-human chorionic gonadotropin, estrogen, progesterone, and placental lactogen. This is particularly interesting considering the difficulty in obtaining large amounts of freshly isolated trophoblast cells and the fact that primary trophoblasts have a short lifetime and hardly proliferate *in vitro* (Hiden et al. 2007). On the other hand, differences have also been described between JAR and JEG-3 cells, especially regarding their proliferative activity,

invasive phenotype and degree of differentiation. JAR cells exhibit higher proliferation rate, higher invasive phenotype and are less differentiated in comparison to JEG-3 cells (Grummer et al. 1994). Doubling time lasts approximately 20 hours for JAR cells and 24 hours for JEG-3 cells. *In vitro*, JAR and JEG-3 cells grow adherent in monolayer or in clusters, and the formation of cell aggregates are particularly common in JEG-3 cells (http://www.dsmz.de).

Hybrid Cell Lines ACH-3P and AC1-M59

ACH-3P and AC1-M59 derive from the hybridization of human trophoblast cells with the choriocarcinoma cell line JEG-3.

ACH-3P cells derive from the fusion of primary human first trimester trophoblasts (week 12 of gestation) with AC1-1 cells, which are HGPRT-negative mutants of JEG-3 cells. ACH-3P cells express trophoblast markers (cytokeratin-7, matrix metalloproteinases, integrins etc) and possess invasive abilities and a transcriptome that closely resemble trophoblasts (Hiden et al. 2007). These properties turn this immortalized cell line into a model of first trimester trophoblast.

AC1-M59 cells are a non-tumorigenic subclone of ACH1-1 cells and derive from the somatic hybridization of AC1-1 cells with term extravillous trophoblast cells (Frank et al. 2000). These cells grow adherent in monolayers *in vitro* and present epithelioid morphology. Doubling time lasts approximately 50 hours (http://www.dsmz.de).

3.2 – Cell Culture

Cells were commercially obtained from ATCC (USA) and were maintained in 175 cm² flasks under standardized conditions (37°C, 5% CO_2, humidified atmosphere) in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2% antimycotic-antibiotic solution, composed of penicillin (100 IU/ml) and streptomycin (100 µg/ml) (AAS; Sigma, Germany). They were observed on a daily basis with an inverse microscope to ensure proper growth and absence of mycoplasma.

Every two or three days, cells were trypsinized and incubated in new medium, in order to avoid disturbances in cell proliferation rate. All procedures were performed under sterile conditions. In these terms, cells were initially washed with PBS and were then incubated with 500 µl trypsin for 5 min under standardized conditions. Upon the action of trypsin, the

adherent cells unstuck from well surface and could be diluted and/or used for experiments. For culture, they were adjusted to 10^5 cells/ml using a Neubauer chamber and were incubated in 2 ml supplemented DMEM medium.

3.3 – Lymphocyte Isolation and Culture

Lymphocytes were isolated using a Ficoll density gradient. Initially, peripheral venous blood of a healthy non-pregnant 25-year old donor was collected and diluted with PBS Buffer (1:1 dilution). Sets of 7 ml diluted blood were carefully layered over 3 ml Ficoll 1,077 in 15 ml Falcon tubes and centrifuged for 20 min at 2500 U/min at room temperature with brake off. Upon this centrifugation, blood elements were separated according to their densities. Erythrocytes and granulocytes, which are denser than the other elements, accumulated at the bottom of the tube, forming a dark red layer. Laying on it, a transpartent layer was identified, corresponding to the Ficoll solution. Between the Ficoll and the upper yellow layer (composed of plasma and PBS), it was possible to identify a thin white layer, composed of peripheral lymphocytes (see Fig. 5). These were gently pipetted from the interface and were mixed with 5 ml RPMI, while erythrocytes and granulocytes were discarded. The solution with lymphocytes was submitted to two wash steps. In the first one, the solution was centrifuged for 8 min at 1500 U/min and the final supernatant was discarded. The pellet was resuspended in 5 ml RPMI and once more centrifuged, now for 5 min at 1500 U/min. The final supernatant was discarded and the remaining pellet was resuspended with 4 ml RPMI Medium (RPMI 1640 with 10% FBS). Cells were then distributed in 2 wells of a 6-well plate and cultured under standardized conditions (37°C in a 5% CO_2 humidified atmosphere).

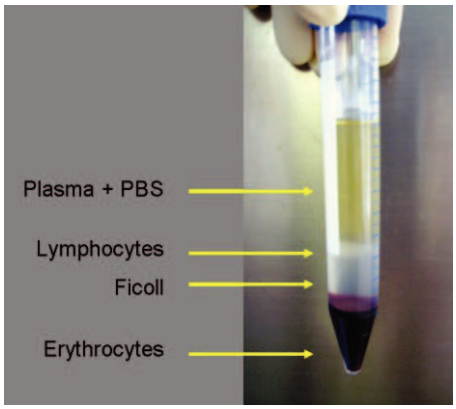
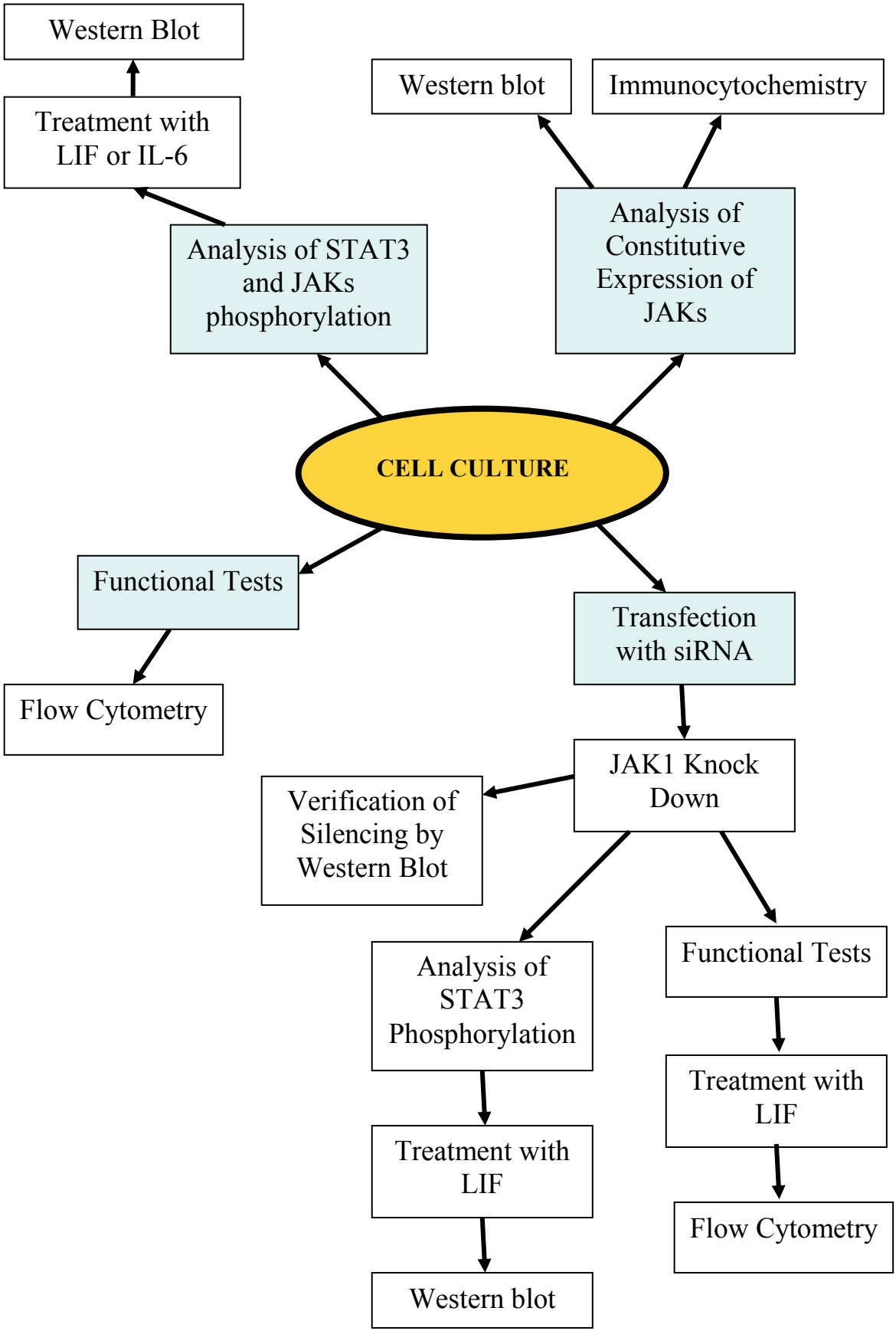


Fig. 05 Lymphocyte isolation

Lymphocytes were then treated with Phytohemagglutinin (PHA) and submitted to cell lyses, as explained forewords, in order to obtain protein samples that could be used as positive control of JAK1, JAK2 and JAK3 expression in the experiments testing the presence of these kinases in choriocarcinoma cell lines.

3.4 – Summary of Experiments



3.5 – Cell Stimulation

Cell lines were treated with Leukemia Inhibitory Factor (LIF) or IL-6 for different periods. After adjusting cells to $1x10^5$/ml with a Neubauer chamber, cells were incubated in wells of a 6-well plate within 2 ml DMEM supplemented with 10% heat-inactivated FBS and with a 2% antimycotic-antibiotic solution, composed of penicillin (100 IU/ml) and streptomycin (100 µg/ml). Cells were prepared in a sterile environment and were maintained under standardized conditions (37°C, 5% CO_2, humidified atmosphere). One day before stimulation, this serum-containing medium was substituted for the same volume of DMEM (2 ml) without FBS or antibiotics, in order to avoid cell stimulation by these factors. At the time of incubation with LIF or IL-6, the cells were approximately 70% confluent. After washing the cells with PBS and adding new 2 ml of pure DMEM, 2 µl of a 10 µg/ml LIF solution were added and gently mixed into each well. In these terms, a solution with approximately 10 ng/ml of LIF concentration was finally achieved. For IL-6 stimulation, 4 µl of a 10 µg/ml IL-6 solution were applied into each well, thus obtaining a solution with final concentration of 20 ng/ml.
Cultured lymphocytes were stimulated with Phytohaemagglutinin (PHA) to a final concentration of 2 µg/ml. PHA is known to stimulate mitotic division of lymphocytes and facilitates chromosome analysis (when cells are arrested in metaphase by addition of colchicine). In addition, PHA treatment has been associated with stimulation of JAKs and STATs in human peripheral blood mononuclear cells (Kilic et al. 1999, Sanchez-Margalet and Martin-Romero 2001). PHA-treated lymphocytes were then submitted to cell lyses, and their protein supernatants were used as positive control in the experiments assessing the JAKs isoforms constitutively expressed in JEG-3 and JAR cells.

3.6 – Cells Lysis and Protein Samples

Cell Lyses

Cell Lysis Buffer (CLB) was used to lyse cells under nondenaturing conditions, aiming to obtain protein samples that would be analyzed with Western blot. After treating cells according to the purpose of the experiment, each well was washed with 1 ml of PBS to remove residual media and given 100 µl of CLB (Cell Signaling) supplemented with 0.1% Protease Inhibitor Cocktail (Sigma). The plates were incubated on ice and cells were then

scraped and put into 1.5 ml labeled Eppendorf tubes, which were later submitted to 3 shock freezing cycles with liquid nitrogen. After frozen, the pellets were gently thawed in ice and then whirled in a vortex equipment. The products were centrifuged at 18,000 rpm, 4°C for 30 min. The final supernatants with proteins were carefully pipetted and stored at –80°C.

Assessment of Protein Concentration

Protein concentrations were assessed by the Bradford Method, which involves the binding of Coomassie Brilliant Blue G-250 dye to proteins. Coomassie forms complexes with cationic and nonpolar hydrophobic protein side chains, what in turn shifts its maximal absorption from 465 nm to 595 nm (Lottspeich and Engels 2006). Protein concentration can thus be inferred measuring the absorption at 595 nm with a spectrophotometer. Experiments were performed as explained: 5 µl of protein lysates or standards (bovine serum albumin with known concentrations - BSA) were mixed with 995 µl of Bradford solution. After a 10-minute incubation period at room temperature in the dark, detection was performed at 595 nm by a spectrophotometer. A standard curve was drawn with the values obtained for BSA, and the protein concentration of the lysates was finally inferred.

3.7 – Protein Analysis

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins according to their molecular weight, as seen in Fig. 06. With the use of sodium dodecyl sulfate (SDS), an anionic detergent, the secondary and non–disulfide–linked tertiary structures of proteins are denatured, leading them to linearize. As it is known, SDS overwhelms proteins with a negative charge, making them to migrate towards the positive pole when placed in an electric field (http://www.ruf.rice.edu/~bioslabs/studies/sds-page/denature.html). Denatured protein

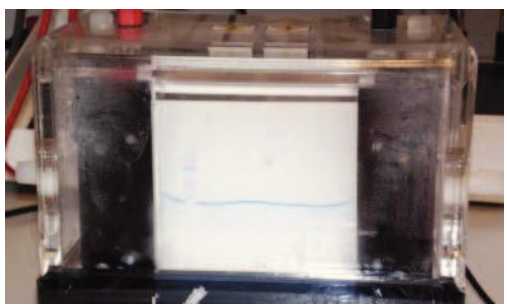


Fig. 06 SDS Electrophoresis

samples are then applied to polyacrylamide gel, a polymer of acrylamide monomers whose structure possesses a multitude of tunnels of different diameters. When the electrical field is applied, proteins start to move through these tunnels towards the anodes. Separation occurs as proteins with smaller molecular weight tend to migrate faster through the gel (Luttmann et al. 2006). In this sense, proteins with the same molecular weight, even with completely different amino acid sequences, travel together in a mixed band.

Electrophoresis and Western Blot

After obtaining the samples and their respective concentrations, proteins were put in the same concentration (20-25 µg/ml) and volume (20-25 µl) and were run applied to 7.5% acrylamid gels for about 1 hour at 30 mA/gel in 500 ml Running Buffer. A protein ladder (dual color standard) was used to determine and control molecular weight. After proteins were fractionated by size, they were electrotransfered to a 10x10 cm nitrocellulose membrane for 38 min at 240 mV (protein blotting), as demonstrated in Fig. 07. Binding depends on hydrophobe interactions and hydrogen bonds among proteins and the membrane (Lottspeich and Engels 2006).

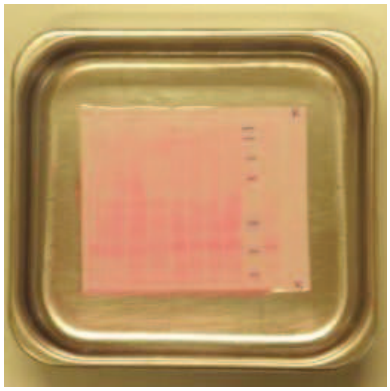


Fig. 07 Proteins on nitrocellulose membrane after electrotransference

Membranes were then blocked in 25 ml of NetG for 30 minutes at room temperature. After that, membranes were incubated overnight at 4°C with the primary antibody (e.g. Phospho-JAK1, JAK2 Antibody etc) at appropriate dilution (usually at 1:1000). On the next day, membranes were washed 3 times during 5 min with NetG and were then incubated for 1 hour with the second antibody (purified goat anti-rabbit IgG conjugated to horseradish peroxidase), diluted at 1:2000 in NetG. After washing 3 times with Wash Buffer, each membrane was covered with plastic wrap and incubated with 1 ml of LumiGLO® for 1 min at room temperature. In order to detect chemoluminescence, membranes were exposed to X-ray films in a dark chamber for different periods (10-35 min, depending on antibody) and films were finally developed for about 10 seconds. Another option to the X-ray exposure was the use of the MF-ChemiBIS 3.2 device, an automated documentation workstation that permits image acquisition (GelCapture). Software for image

analysis was used to detect and quantify protein bands (TotalLab TL100, version 2006, Nonlinear Dynamics Ltd.).

3.8 – RNA Interference (RNAi)

RNA interference (RNAi) is a method to knock down gene expression post-transcriptionally, permitting to investigate gene and protein function in different cell types (Fire et al. 1998, Wall and Shi 2003). It constitutes an evolutionary highly conserved process firstly observed in plants in the early 1990s, which was subsequently identified in fungus, invertebrates and mammals (Hannon 2002, Hamilton and Baulcombe 1999). In 1998 it was reported its underlying mechanism, in which double-stranded RNAs (dsRNA) were demonstrated to silence targeted genes in *Caenorhabditis elegans* (Fire et al. 1998). Since then, the process of gene silencing has been intensively investigated and regarded as a precious research tool, by which specific genes can be shut down and their role in several cellular processes can be analyzed. In humans, RNAi has been demonstrated to play a role in maintenance of genome integrity, cell defense against viral infection and regulation of gene expression (Sioud 2005).

In cell research, traditionally long dsRNAs are delivered into the cell by lipid-mediated transfection or by viral-mediated transduction, depending on cell type and desired knock-down period (transient or stable). The dsRNA is then cleaved intracellularly by the enzyme Dicer, an ATP-dependent ribonuclease III enzyme with helicase activity (Bernstein et al. 2001). The dsRNA is processed into short fragments of 20-25 base pairs (sense and antisense strands) with 2-nucleotide 3'-overhangs and 5'-phosphorylated terminus, known as small interfering RNAs (siRNA) (Bernstein et al. 2001, Hutvagner and Zamore 2002). Subsequent to unwinding, the sense strand is cleaved and degraded, whereas the antisense strand is incorporated by the RNA-induced silencing complex (RISC), which detains endonuclease, exonuclease, helicase and homology-searching domains (Sledz and Williams 2005, Hannon 2002).

After incorporation, complementary sequences in mRNA molecules are then screened by these complexes. When paired, the targeted mRNA is cleaved by argonaute (a catalytic component of RISC) into portions that cannot be translated (Zeng et al. 2003). The mechanism of RNA interference is summarized in Fig. 08.

Instead of using long dsRNAs, which have been reported to activate the innate immune response in mammalian cells (Williams 1999), it is also possible to achieve RNAi by transfection of synthesized 21-23 base pair siRNA duplexes. These duplexes mimic the

products originated by the action of Dicer on dsRNAs. After delivery in the cell, the sense and antisense strands of siRNA duplexes are processed similarly to dsRNAs. The antisense strand is incorporated by RISC and pairs with targeted mRNAs, while the sense strand is degraded (Elbashir et al. 2001, Caplen et al. 2001).Additionally, RNAi can be triggered by synthetic siRNAs, as well as by microRNAs (miRNAs), which are physiologically transcribed from the genome. As previously described, RNAi permits the post-transcriptional suppression of specific genes, ultimately contributing to a better comprehension of the components necessary for particular processes, such as cell division, migration and invasion.

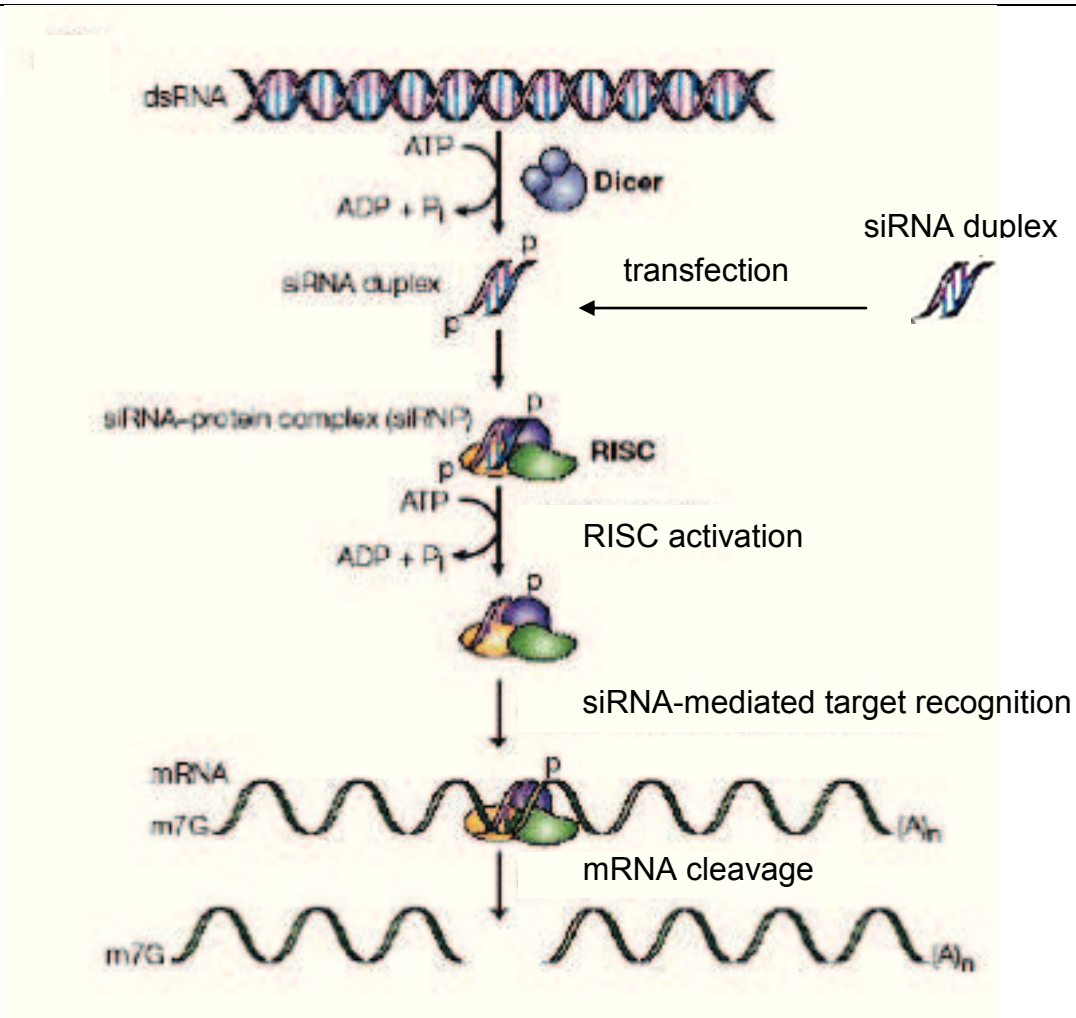


Fig. 08 Mechanism of RNA interference (Modified from Dykxhoorn 2003)

Long double-stranded RNA (dsRNA) is digested by Dicer in siRNAs, which are then uptaken by RISC. Optionally, synthesized siRNA duplexes, which mimic the products originated by the action of Dicer on dsRNAs, may be delivered into the cell by transfection The single-stranded antisense strand helps RISC to find the target mRNA, culminating in specific mRNA cleavage and post-transcriptional gene silencing (Dykxhoorn et al. 2003).

Oligonucleotide Annealing

In order to obtain double-stranded RNAs (dsRNAs), designed to target JAK1, the same amount of single sense and anti-sense strands were diluted in nuclease-free water and were put together in 5x Annealing buffer, finally obtaining a mixture at a final concentration of 10 µM. This mixture was then incubated for 1 min at 95° C and subsequently for 1 h at 37° C. This process facilitates single RNA strands to pair by hydrogen bonds to their complementary sequences, forming double-stranded oligonucleotides. Annealed oligonucleotides were then aliquoted and stored at -20° C.

Cell Transfection

After entering the cell, the dsRNA can be degraded by dicer into siRNA, which will ultimately promote specific gene silencing. But before achieving the intracellular space, the negatively charged dsRNA must pass across the lipid bilayered cell membrane. Several mechanisms have been developed to promote the dsRNA uptake into the cell, a process usually referred to as cell transfection and that has been considered a crucial step for the subsequent gene silencing. Two reagents have been used in this work for transfection – Oligofectamine and Nanofectin.

Transfection with Oligofectamine

Oligofectamine is a cationic lipid-based reagent that spontaneously interacts with oligonucleotides, forming transfection complexes that facilitate the delivery of antisense oligonucleotides and dsRNAs into the cells (Li et al. 2002, Elbashir et al. 2001)Oligofectamine™ Reagent datasheet 2004). All experiments using Oligofectamine were performed according to the Protocol for siRNA Transfection of the Placenta Laboratory. Cells were initially trypsinized and split about 30-40% confluent in 6-well plates in 2 ml of DMEM supplemented with 10% heat-inactivated FBS and 2% antimycotic-antibiotic solution. After overnight incubation under standardized conditions (37°C, 5% CO_2, humidified atmosphere), the medium was aspirated and cells were washed with 500 µl of OPTIMEM. 800 µl of fresh OPTIMEM were then added to each well, and the "Oligos-Oligofectamine" mix was prepared as follows: a solution composed of 175 µl of OPTIMEM and 10 µl of Oligos (i.e. Scrambled sequence or JAK1 siRNA, from 10 µg/µl aliquots) was mixed with 11 µl of OPTIMEM and 4

µl of Oligofectamine. After a 20-minute incubation period at room temperature, this mix was carefully added to the cells, which were subsequently incubated for 4 hours under standardized conditions. In order to stop RNAi transfection, 500 µl of DMEM containing 30% of heat-inactivated FBS and no antibiotic were given to each well. According to their purpose, cells were incubated for different periods (24 or 48h) and stimulated with LIF or not. Finally the cells were submitted to lyses as previously described, and lysates were used in electrophoresis and Western Blot. Scrambled siRNA with a non-genomic sequence was used as control to verify possible unspecific effects intrinsic to transfection.

Transfection with Nanofectin

Nanofectin is a transfection reagent based on nanotechnology. It consists of nanoparticle-based compounds and small positively charged carrier molecules, which are supposed to facilitate siRNA binding and transference into mammalian cells (Nanofectin & Co datasheet). In this work, all experiments were performed according to the Protocol for siRNA Transfection of the Placenta Laboratory. Cells were initially trypsinized and split about 50% confluent in 6-well plates in 2 ml of DMEM supplemented with 10% heat-inactivated FBS and 2% antimycotic-antibiotic solution. After overnight incubation under standardized conditions (37°C, 5% CO_2, humidified atmosphere), the medium was aspirated and cells were washed with 500 µl of OPTIMEM. 800 µl of fresh OPTIMEM (without serum or antibiotic) were then added to each well, and the Nanofectin mix was prepared. Considering that Nanofectin-mediated transfection had not been tested so far for JAK1 gene silencing in our laboratory, four mixtures with different concentrations of JAK1 siRNA and Nanofectin have been prepared, aiming to obtain a mix with optimal silencing rates. In this sense, solutions composed of 60 µl of OPTIMEM and 6 or 9 µl of JAK1 siRNA (from 10 µg/µl aliquots) were mixed with 60 µl of OPTIMEM and 10 or 15 µl of Nanofectin. The mix containing the Scrambled siRNA sequence was prepared as follows: a solution composed of 60 µl of OPTIMEM and 6 µl of Scrambled siRNA (from 10 µg/µl aliquots) was mixed with 60 µl of OPTIMEM and 10 µl of Nanofectin. After a 20-minute incubation period at room temperature, the resultant mixtures were carefully added to the cells, which were subsequently incubated for 4 hours under standardized conditions. In order to stop siRNA transfection, 500 µl of DMEM containing 30% of heat-inactivated FBS and no antibiotic were given to each well. After a 24-hour incubation period, cells were finally submitted to lyses as previously described, and lysates were used for Western Blot. Scrambled siRNA, which contains a non-

genomic sequence, was used as control to verify possible unspecific effects intrinsic to transfection.

3.9 – Functional Assays

In order to test the hypotheses that Leukemia Inhibitory Factor (LIF) modifies functional parameters of JAR cells, it was performed a set of experiments assessing the proliferative, migratory and invasive behavior of these cells after LIF treatment, as explained as next. Results were compared with the behavior of untreated cells. Furthermore, it was investigated if the disruption of Janus kinase 1 (JAK1) through RNA interference might alter cell behavior. In this context, JAK1 knock-down was performed using a specific siRNA sequence and a new set of functional assays was conducted. Finally, JAK1-silenced cells were stimulated with LIF to verify if this cytokine might modify cell behavior, even after disrupting a component of its signaling cascade.
All experiments started with about 30,000 cells (value extrapolated after cell counting with a Neubauer chamber), which were seeded into individual wells of a 24-well plate (Fig. 09). In those experiments in which LIF was used, cells were stimulated throughout the cultivation period (24 hours) at a final concentration of 10 ng/ml. Final cell counting was performed by flow cytometry.

Preparation of *in vitro* Cell Proliferation Assay

Cells were initially prepared according to the specific goal of the experiment (for example, in the experiments testing the behavior of JAK1-silenced cells, RNAi was initially performed using a specific siRNA sequence and Oligofectamine for transfection, as previously described). Cells were then trypsinized and counted with the use of a Neubauer chamber. 30,000 cells were commenced into wells of a 24-well plate in a final volume of 700 µl of DMEM with nor serum or antibiotic. When possible effects of LIF were tested, LIF was applied into each well at a final concentration of 10 ng/ml. Plates were incubated during 24 hours under standardized conditions (37°C, 5% CO_2, humidified atmosphere). On the next day, the supernatant was initially transferred into FACS tubes. Each well was then washed with 200 µl of PBS, which was also collected in the tubes. Each well was trypsinized (200 µl

of Trypsin), and washed once more with new 200 µl of PBS. All these solutions were added into the FACS tubes for subsequent assessment of proliferation by analysis of cell number.

Preparation of *in vitro* Cell Migration Assay

Firstly the cells were prepared according to the specific goal of the experiment (e.g. JAK1 knock-down). They were then trypsinized and counted with the use of a counting chamber. About 30,000 cells were put in a final volume of 300 µl of serum-free DMEM and were applied onto filters of 24-well plate size (upper well). Each filter was laid down on a well of the 24-well plate (lower well) previously filled with 400 µl of DMEM without FBS or antibiotics. When possible effects of LIF stimulation were tested, LIF was applied and gently mixed into the lower well at a final concentration of 10 ng/ml. The plate was incubated during 24 hours under standardized conditions (37°C, 5% CO_2, humidified atmosphere). On the next day, the medium beyond the filter was initially transferred into FACS tubes. Each well was then washed with 200 µl of PBS, which was also collected in the tubes. Each well and the respective downside of the filter were trypsinized (200 µl of Trypsin), and washed once more with new 200 µl of PBS. All these solutions were added into the FACS tubes for subsequent counting.

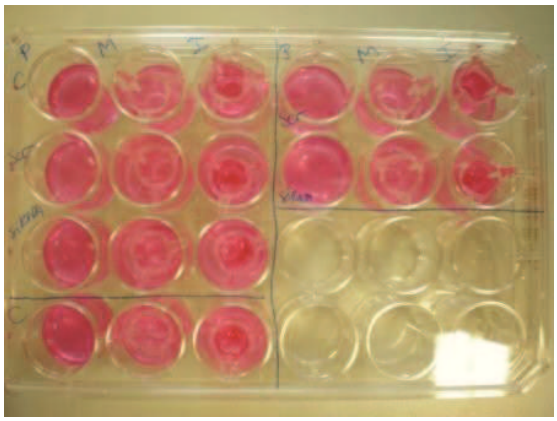


Fig. 09 Proliferation (P), migration (M) and invasion (I) assays performed in duplicate. Note that filters have been placed on wells for migration and invasion tests (2nd, 3rd, 5th and 6th columns)

Preparation of *in vitro* Cell Invasion Assay

Initially the Matrigel™ solution (1:10 dilution in pure DMEM media) was prepared and 100 µl were applied onto filters of 24-well plate size, and let to dry in incubator for 4 hours. The remaining liquid was carefully pipetted. The cells that had been previously prepared according to the specific goal of the experiment (e.g. JAK1 knock-down) were trypsinized and counted with the use of a counting chamber. 30,000 cells were then put in a final volume

of 300 µl of serum-free DMEM and were applied onto the gel. Each filter containing the gel and the cells was laid down on a well (of the 24-well plate) previously filled with 400 µl of DMEM with no supplements. When possible effects of LIF stimulation were tested, this cytokine was applied and gently mixed on the solution on the gel in a final concentration of 10 ng/ml. The plate was incubated during 24 hours under standardized conditions (37°C, 5% CO_2, humidified atmosphere). On the next day, the medium beyond the filter was initially transferred into FACS tubes. Each well was then washed with 200 µl of PBS, which was also collected in the tubes. Each well and the respective downside of the filter were trypsinized (200 µl of Trypsin), and washed once more with new 200 µl of PBS. All these solutions were added into FACS tubes for analysis.

3.10 – Flow Cytometry

This technique was used to count cells in the proliferation, migration and invasion assays. The flow cytometer possess a fluidic system that forces a sample of cells to pass in a single cell stream through a laser beam, in a way that each cell scatters light or emits fluorescence. An optic system composed of several forward and perpendicular scatters and fluorescent detectors collect these light signals, which are then converted into numerical values that can be analyzed by a designed software. The parameters measured by this optic system permits deriving data about the chemical and physical structure of individual cells, which can be finally counted and characterized. Furthermore, monoclonal antibodies directed toward surface antigens and conjugated with fluorescent molecules can be used to label or tag cells. Fluorescent signals emitted when the cells are funneled to the laser beam are in the same way collected by the optic system, making cell sorting possible (fluorescence activated cell sorting). Several parameters may be assessed by flow cytometry, including cellular phenotype, DNA and RNA content, cell viability and granularity etc (BD Biosciences datasheet 2008).

In the set of experiments, the tubes of the flow cytometer were prepared as described in *in vitro* cell proliferation, migration and invasion assays. Prior to analysis, a mixture of 100 µl of PBS and Polystyrene Beads (TrueCount Beads) was added to each tube. Bead number was also quantified during the flow cytometry tests. This parallel bead counting served as control, assuring the cytometer to stop cell counting when a predetermined number of beads had been

reached. Beads and cells were differentiated by the cytometer through their different size, as evidenced in the dot plot graphic in Fig 10.

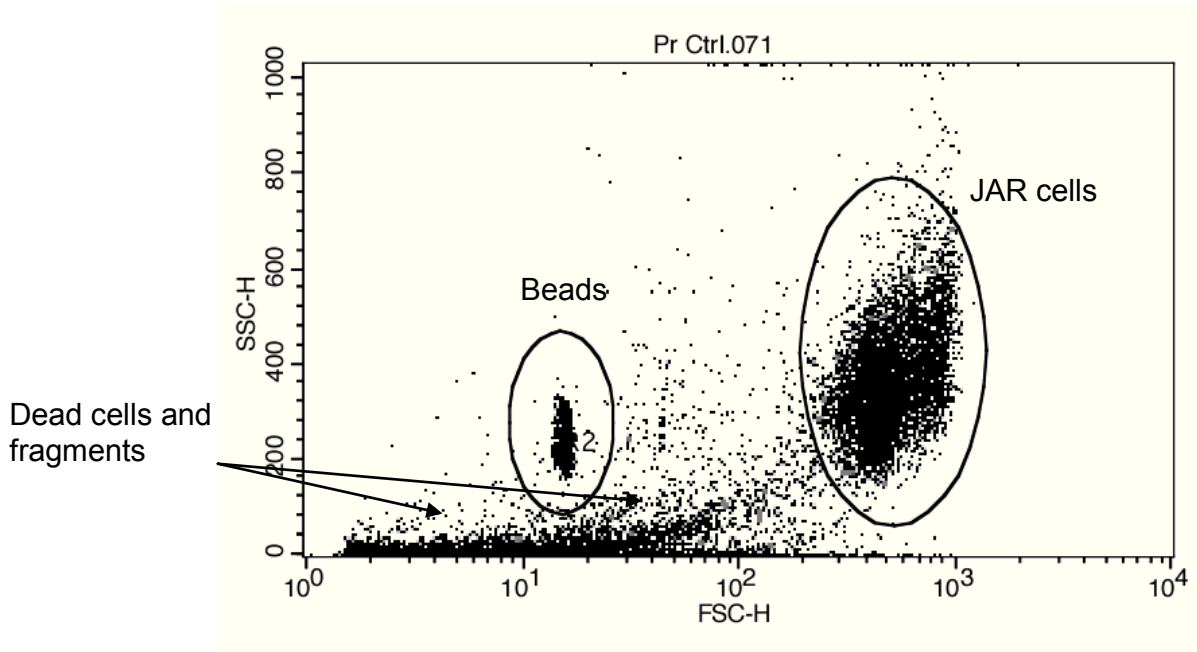


Fig. 10 Flow Cytometry Dot-Plot

Flow Cytometry was used to quantify JAR cells in the functional assays. Predetermined volumes of cell suspension were incubated with polystyrene beads. Cells and beads were differentiated by the Flow Cytometer according to their sizes. Cell counting has stopped when a predetermined number of beads had been detected, serving as control. (FSC-H = Forward Scatter; SSC-H = Sideward Scatter)

3.11 – Immunocytochemistry

Immunocytochemical studies were performed to identify the constitutive expression of Janus Kinases 1, 2 and 3 in JAR, JEG-3 and human placental cells. Placentary tissues have been obtained from a healthy woman who underwent elective abortion in the 11th week of pregnancy. These tissues were kindly prepared by Maja Weber (Ph.D. student). Immunocytochemical staining protocol was defined by Maja Weber and Prof. Sebastian San Martin.

Placental tissues were initially washed with PBS and incubated during 48 hours at 4°C in 4% Paraformaldehyde in 0.1 M PBS for cell fixation. Cells were washed with 0.1 M glycine in PBS overnight at 4°C. On the next day they were submitted to an ethanol-based hydration, in

which cells were incubated with crescent concentrations of ethanol (50%, 70%, 95% and 100% during 30 min for each concentration) at room temperature. Incubation with 95% and 100% ethanol was repeated three times. After washing ethanol three times (15 min each) with Xylol, the tissue was imbibed with paraffin (Histosec) four times (30 min each) at 56-60°C. 5 µm thick tissue slices were obtained by a microtome. These slices were laid down on microscope slides and were stored at 4°C. In order to deparaffinize and rehydrate the samples, slides were incubated twice in Xylol for 30 min each time, followed by ethanol incubation (twice in 100% ethanol for 30 min each time, then once in 95% for 30 min and once more in 70% ethanol for 30 min) and finally in distilled water for 15 min. Subsequently the tissue slices were incubated in 10 mM citrate sodium buffer (pH 6.0) for 10 min at 95°C and cooled down during 30 min in the same buffer for antigenic retrieval. They were then washed three times with distilled water for 5 min each time, followed by wash with 0.1M PBS twice for 15 min. For unspecific binding blocking the slides were incubated during 1 hour in 5% goat serum diluted in 0.3% PBS/Tween20 at room temperature in a humid chamber. Final blocking was obtained with incubation in Cas-Block (Zymed) for 10 min. The selected first antibody (Jak1, Jak2 or Jak3 antibody) diluted 1:100 in PBS/Tween20 was then applied on the fixed tissue and slides were incubated overnight at 4°C. On the next day, the slides were washed tree times with 0.1 M PBS for 5 minutes and incubated with the second antibody (Anti-Rabbit IgG, which is conjugated to Fluorochrome Cy3) during 1 hour in a 1:400 0.1 M PBS solution at room temperature. Slides were washed three times with 0.1 M PBS and the cell nucleus were mounted with 1.5 µg/ml DAPI medium (4,6-Diamidino-2-phenylindole-dihydrochloride). A cover slip was laid on each slide, which was finally sealed with nail enamel (Yves Saint Laurent Paris, France). Slides were stored at 4° C and a fluorescence microscope was used for analysis.

JAR and JEG-3 cells suspended in DMEM were separately applied onto Poly-L-Lysine covered microscope slides after 1:1000 dilution in GBSS (Gey's Balanced Salt Solution). The slides were incubated overnight under standardized conditions (37°C, 5% CO_2, humidified atmosphere) and applied on the next day to 4% Paraformaldehyde during 1 hour for cellular fixation. After washing twice with 0.1 M PBS during 15 minutes, the slides rested during 1 hour in 5% goat serum diluted in 0.3% PBS/Tween20 at room temperature in a humid chamber. Final blocking to avoid unspecific binding was reached with incubation in Cas-Block (Zymed) for 10 min. The selected first antibody (Jak1, Jak2 or Jak3 antibody) diluted 1:100 in PBS/Tween20 was then applied on the fixed cells and slides were incubated overnight at 4°C. On the next day, the slides were washed tree times with 0.1 M PBS for 5

minutes and incubated with the second antibody (Anti-Rabbit IgG) in a 1:400 0.1 M PBS solution during 1 hour at room temperature. Slides were washed three times with 0.1 M PBS and the cell nucleus were mounted with 1.5 µg/ml DAPI medium (4,6-Diamidino-2-phenylindole-dihydrochloride). A cover slip was laid on each slide, which was finally sealed with nail enamel. Slides were stored at 4° C until analysis with fluorescence microscope.

3.12 – Documentation and Significance Tests

Western blot pictures were obtained using the Gel Documentation System, composed of the equipment MF-ChemiBIS 3.2 and the software Gel-Capture. Quantitative analysis of Western blot bands was performed using the softwares Gel-Capture and TotalLab 100 Version 2006 (Nonlinear Dynamics Ltd).

Flow cytometry experiments were conducted using the equipment FACSCalibur (BD Bioscience). Their qualitative and quantitative results were assessed by the Cell Quest Pro Program.

Bars, graphics, tables and statistical analysis were prepared, calculated and/or presented using Microsoft Excel.

Error bars indicate, according to the experiment, standard deviation (S.D.) or standard error (S.E.), which was calculated to consider the range of error factors. Significance was determined by Student's T-test, which calculates the probability (p) of two data sets to be not significantly different from each other. The difference was considered significant when $p < 0.05$.

4. Results

4.1 – LIF and IL-6 Influence on STAT3 Phosphorylation

In order to investigate if Leukemia Inhibitory Factor (LIF) and Interleukin-6 (IL-6) are associated with the phosphorylation of tyrosine STAT3, different cell lines (JEG-3, JAR, AC1-M59 and ACH-3P) were cultured and stimulated with LIF or IL-6 for different periods at final concentrations of 10 ng/ml and 20 ng/ml respectively. At the time of LIF or IL-6 treatment, cells were approximately 70% confluent in 6-well plates filled with 2 ml of pure DMEM. After each desired stimulation time has been reached, cell lyses and Western blot were performed for protein analysis.

The figure below (Fig. 11.1) evidences STAT3 phosphorylation using Western blot. As it can be observed, very intense bands of phosphorylated tyrosine 705 STAT3 (Ph-STAT3) were verified in cell groups stimulated with LIF. Practically no bands for Ph-STAT3 are seen in respective control groups and cells that were treated with IL-6. ß-Actin was used as control. Cells were stimulated for 10 min (except Control group). These findings are supported by the quantification of Western blot bands, seen as next (Fig. 11.2). Values were normalized to control of each cell group, which value was set as 100%. In the three cell groups, stimulation with LIF was associated with a significant increase of phosphorylated STAT3 ($p<0.05$, $n=4$).

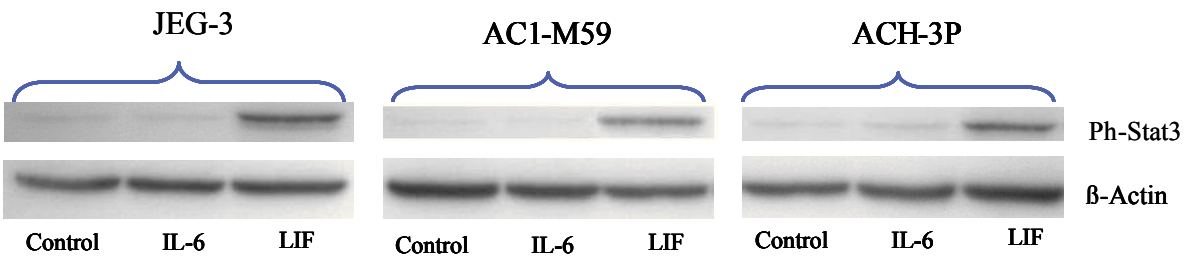


Fig. 11.1 STAT3 tyrosine phosphorylation after IL-6 and LIF stimulation in JEG-3, AC1-M59 and ACH-3P cells (Western blot bands) (n=4)

Cell groups: Control (non-treated cells), IL-6 (IL-6 treated cells), LIF (LIF-treated cells).

Ph-STAT3 (Tyr705): phosphorylated tyrosine 705 STAT3.

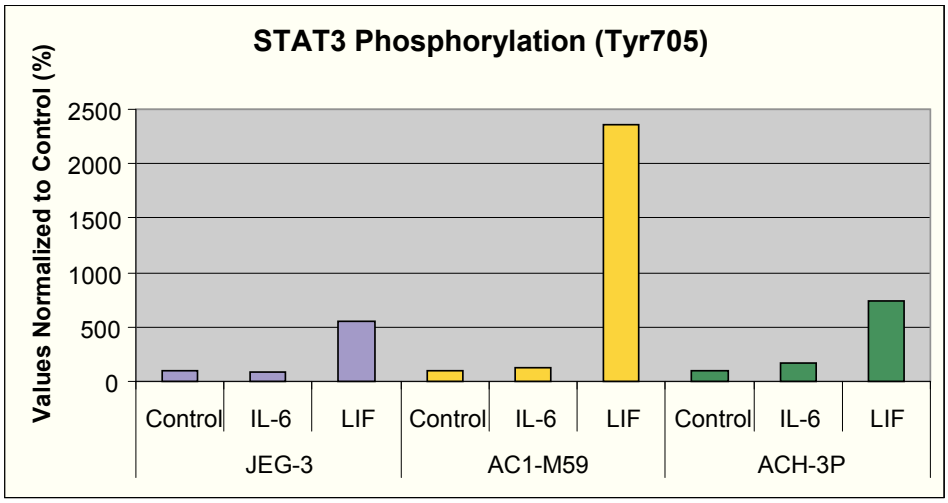


Fig. 11.2 STAT3 tyrosine phosphorylation after LIF or IL-6 stimulation in JEG-3, AC1-M59 and ACH-3P cells (quantification of Western blot bands)

Cell groups: Control (non-treated cells), IL-6 (IL-6 treated cells), LIF (LIF-treated cells)

Subsequently, STAT3 tyrosine phosphorylation was assessed in JEG-3 and JAR cells after different periods of LIF stimulation. Treatment length ranged from 5 to 40 min in JEG-3 cells and from 02 to 10 min in JAR cells. As demonstrated in Fig. 12, phosphorylated STAT3 was detected during the whole experiment after LIF stimulation in JEG-3 and JAR cells. ß-Actin was used as reference control.

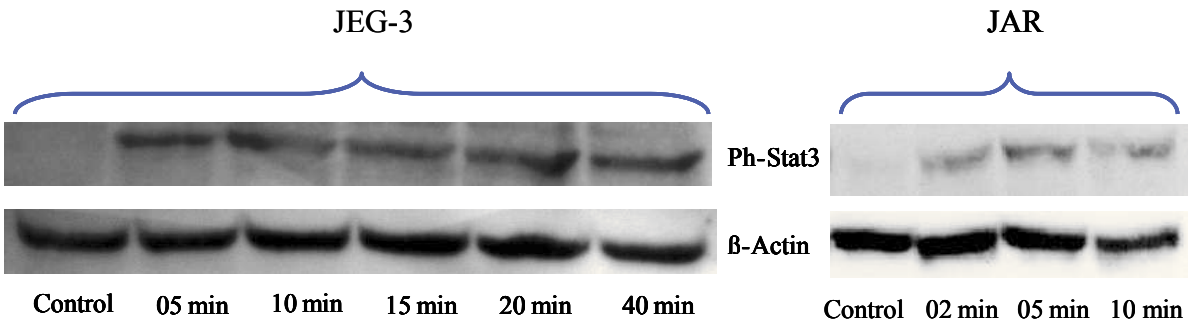


Fig. 12 STAT3 tyrosine phosphorylation induced by LIF stimulation in JEG-3 and JAR cells (n=2)

Cell groups: Control (non-treated cells). 05 min, 10 min, 15 min, 20min and 40 (LIF-treated cells. Treatment length ranged from 5 to 40 minutes).

Ph-STAT3: phosphorylated tyrosine 705 STAT3.

The Western blot bands below (Fig. 13.1) demonstrate another evidence of STAT3 phosphorylation. In these experiments, JEG-3 and JAR cells were treated with LIF for different periods (treatment length ranging from 2 to 10 min), except Control group. Phosphorylated and non-phosphorylated STAT3 were assessed. As it can be observed, phosphorylated STAT3 bands (Ph-STAT3) get more intense with longer periods of LIF stimulation. On the other hand, the expression of non-phosphorylated STAT3 becomes progressively reduced in both cell groups, suggesting that LIF induces the conversion of the non-phosphorylated STAT3 into it activated form. The quantification of these Western blot bands reinforces this evidence, as seen in Fig. 13.2. Values were normalized to STAT3 expression (each value derives from the ratio Ph-STAT3 / STAT3 using the values obtained from Western blot bands quantification). Values of control groups (in which cells had not been incubated with LIF) were set as 100%. With longer periods of LIF treatment, the ratio above indicates that STAT3 is being progressively converted into its phosphorylated form.

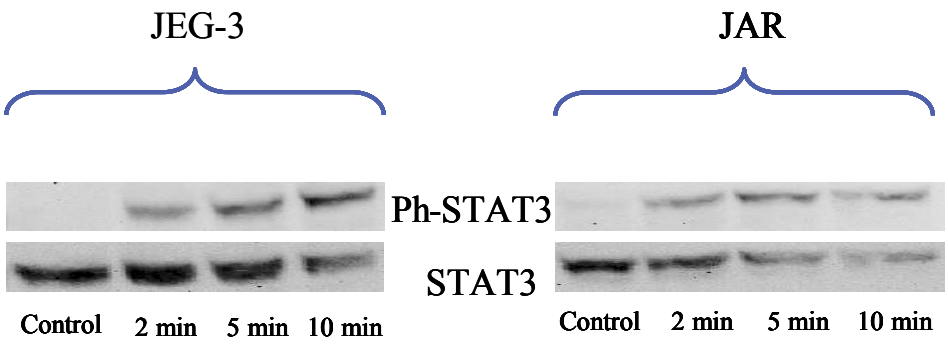


Fig. 13.1 STAT3 phosphorylation (Tyr705) after LIF stimulation in JEG-3 and JAR cells (n=2)

Cell groups: Control (non-treated cells). 2 min, 5 min and 10 min (LIF-treated cells. Treatment length ranged from 2 min to 10 min).

Ph-STAT3: phosphorylated tyrosine 705 STAT3; STAT3: non-phosphorylated STAT3.

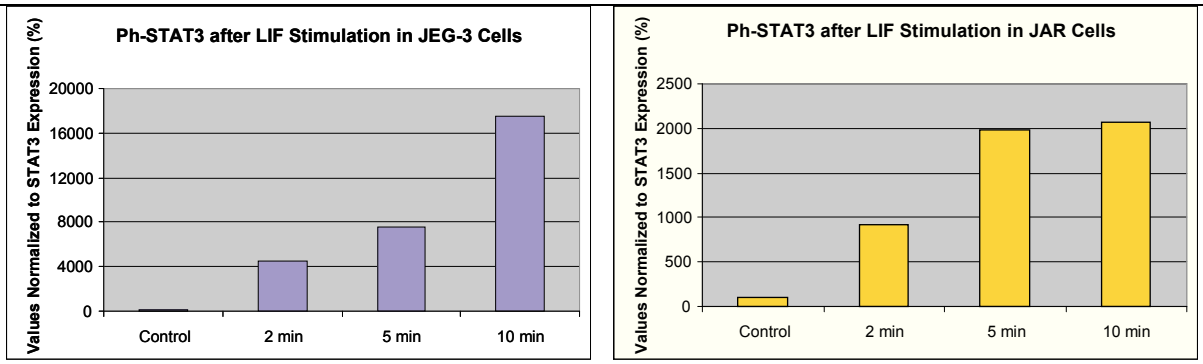


Fig. 13.2 STAT3 phosphorylation (Tyr705) after LIF stimulation in JEG-3 and JAR cells (quantification of Western blot bands)

Cell groups: Control (non-treated cells). 2 min, 5 min and 10 min (LIF-treated cells. Treatment length ranged from 2 min to 10 min).

4.2 – Constitutive Expression of Janus Kinases in JEG-3 and JAR cells and Their Possible Involvement in the LIF-Induced STAT3 Tyrosine Phosphorylation

Different Janus Kinases (JAK1, JAK2 and JAK3) were initially assessed in JEG-3 and JAR cells, aiming to know which isoforms might be constitutively expressed in these cells. In order to investigate if LIF is associated with the phosphorylation of these JAK isoforms, cells were stimulated with LIF for different periods at a final concentration of 10 ng/ml. At the time of LIF incubation, cells were approximately 70% confluent in 6-well plates filled with 2 ml of pure DMEM. After each desired stimulation time was reached (ranging from 2 to 10 min), cell lyses and Western blot were performed.

The next figure (Fig. 14) shows Western blot bands obtained from experiments assessing the constitutive isoforms of Janus Kinases expressed in JEG-3 and JAR cells. The rectangles show the bands obtained in control groups (i.e. cells that had not been stimulated with any cytokine). Lymphocytes stimulated with PHA (Lymph + PHA) were used as positive control for the three tested JAKs isoforms. ß-Actin was used as reference control. Comparing the red rectangles, it can be observed that JAK1 is constitutively expressed in JAR cells, but not in JEG-3 cells (or maximally to a very marginal degree). Taken the orange rectangles, an intense signal of JAK2 antibody can be seen in JAR cells, but just a very slight signal in JEG-3 cells. Considering the yellow rectangles, it can be observed that JAK3 is not constitutively expressed in these cells. Taken these data together, it can be inferred that JEG-3 cells express

mainly JAK2 constitutively, but only to a marginal degree, whereas JAR cells constitutively express JAK1 and JAK2.

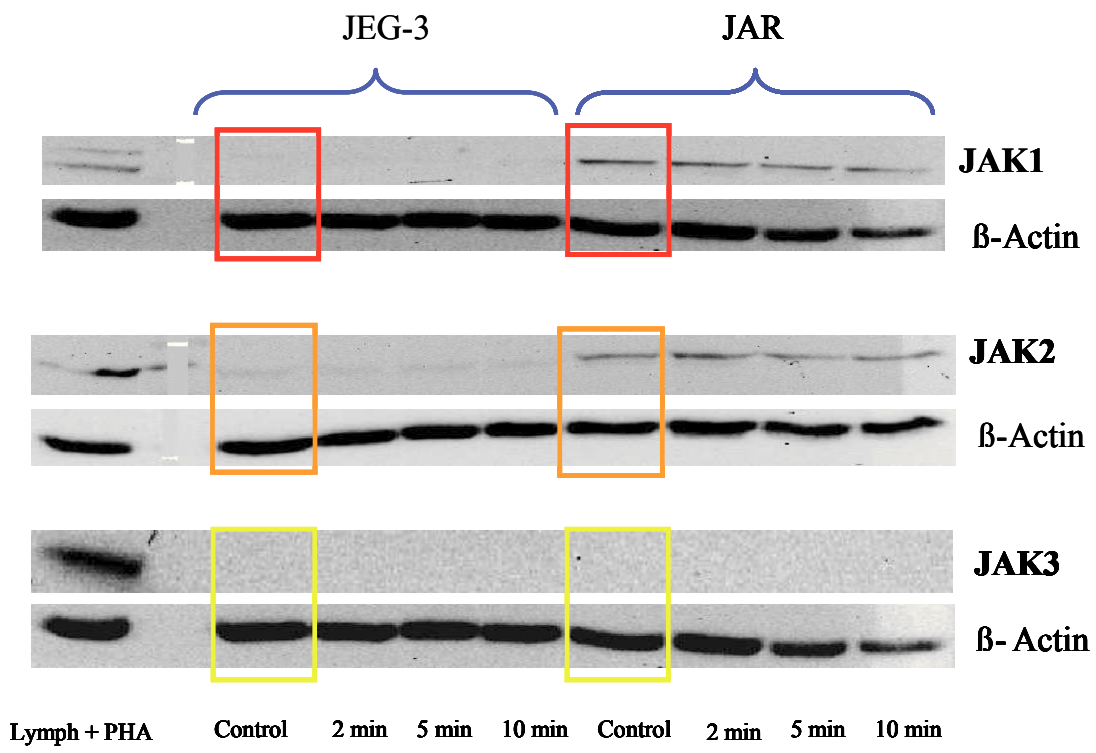


Fig. 14 Constitutive expression of JAK1, JAK2 and JAK3 in JEG-3 and JAR cells (n=3)
Cell groups: Lymph + PHA (lymphocytes stimulated with PHA); Control (non-treated cells); 2 min, 5 min and 10 min (LIF-treated cells. Treatment length ranged from 2 to 10 minutes). JAK1: constitutively expressed JAK1; JAK2: constitutively expressed JAK2, JAK3: constitutively expressed JAK3

4.3 – Constitutive Expression of Janus Kinases in Choriocarcinoma Cell Lines and Placental Tissues Assessed by Immunocytochemistry

Immunocytochemical Analysis in JEG-3 and JAR Cells

Immunocytochemical studies were performed to proof the results obtained by Western blot, concerning the JAK isoforms constitutively expressed in JEG-3 and JAR cells. After 24-hour incubation, cells were fixed with paraformaldehyde and were then maintained with the first (JAK1, JAK2 or JAK3) and second antibodies, according to protocol. Images are seen below (Fig. 15 and 16).

The images obtained from the immunocytochemical experiments evidence, in most cases, similarities with the results obtained by Western blot. In JEG-3 cells, JAK2 antibody promoted an intense and diffuse staining of cell membrane and cytoplasm. JAK1 staining was practically negligible, while JAK3 staining was absent. In JAR cells, all JAK antibodies correlated with staining of cell membrane and cytoplasm. However, staining was much more evident for JAK1, not so intense for JAK2 and practically insignificant for JAK3.

JEG-3 Cells

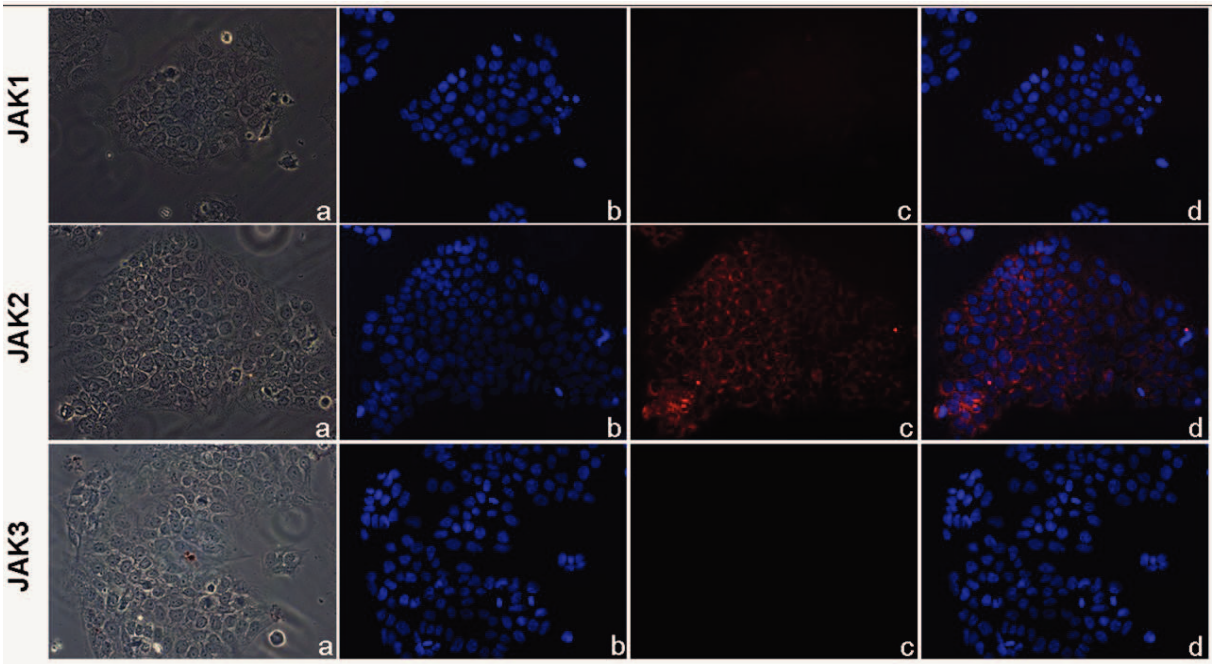


Fig. 15 Constitutive expression of JAK1, JAK2 and JAK3 in JEG-3 cells (n = 1)

a = cells in phase contrast imaging; b = cell nucleus (DAPI stain); c = JAK isoform staining; d = addition of b + c. Magnification (x 200).

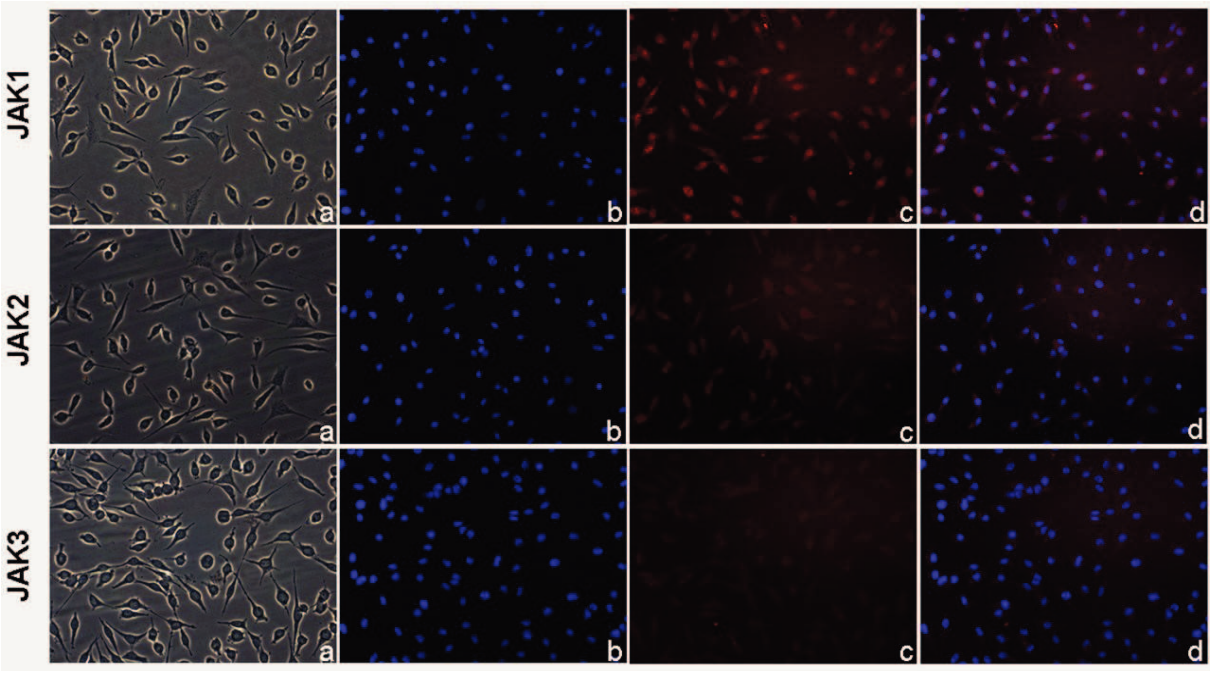


Fig. 16 Constitutive expression of JAK1, JAK2 and JAK3 in JAR cells (n = 1)

a = cells in phase contrast imaging; b = cell nucleus (DAPI stain); c = JAK isoform staining; d = addition of b + c. Magnification (x 200).

Immunocytochemical Analysis in Placental Tissues

Immunocytochemical analysis was performed to assess which JAK isoforms are constitutively expressed in placental tissues. Samples were obtained from the placenta of a healthy woman undergoing elective abortion during the 11th week of pregnancy. JAK expression was then compared to that observed in choriocarcinoma cell lines. After 24-hour incubation, villous and extravillous trophoblast cells were fixed with paraformaldehyde and were then maintained with the first (JAK1, JAK2 or JAK3) and second antibodies, according to protocol. Images are seen below (Fig. 17 and 18).

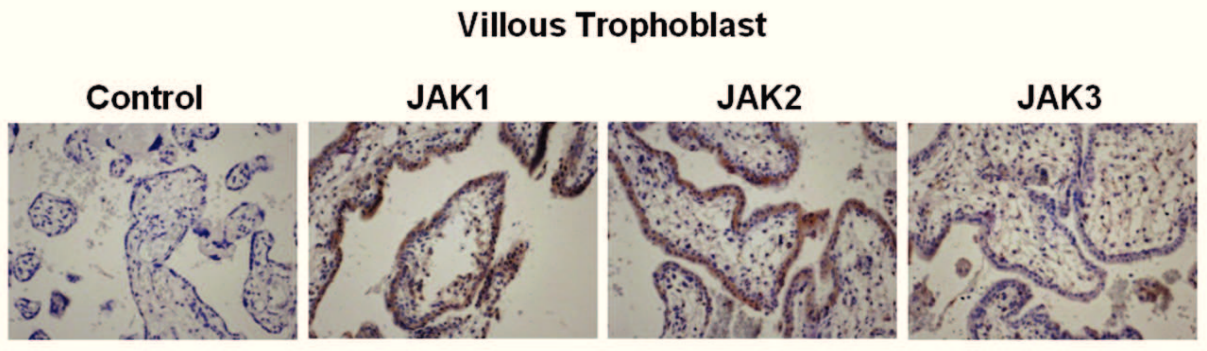


Fig. 17 Constitutive expression of JAK1, JAK2 and JAK3 in fist trimester villous trophoblast cells (n=1)

Immunocytochemical analysis identified strong signals of JAK1 and JAK2 in the periphery of placental villi, mostly in the cytoplasm. Practically no JAK3 staining was verified. Magnification (x 100).

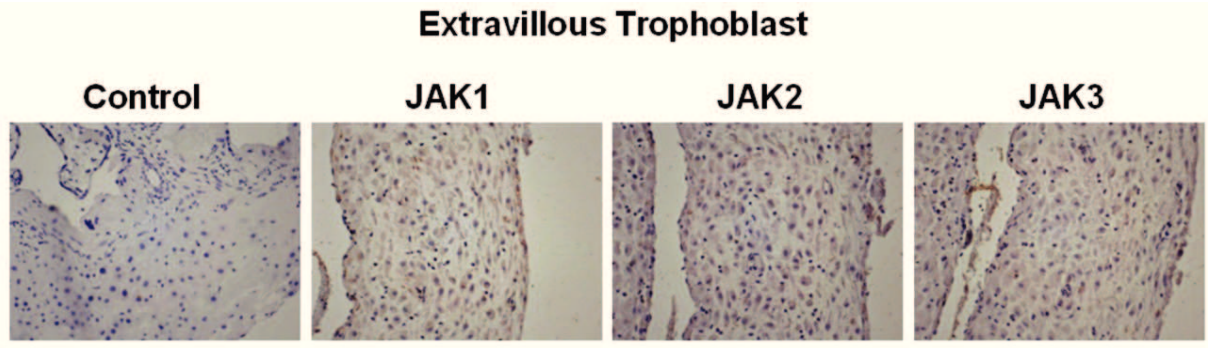


Fig. 18 Constitutive expression of JAK1, JAK2 and JAK3 in first trimester extravillous trophoblast cells (n=1)

Immunocytochemical analysis revealed just a marginal degree of JAK1, JAK2 and JAK3 staining in extravillous trophoblast cells, mostly in the cytoplasm. Magnification (x 100).

4.4 – LIF-Induced Janus Kinases Phosphorylation in JEG-3 and JAR cells

Janus Kinase 1

The Western blot bands below (Fig. 19) derive from experiments in which JEG-3 and JAR cells were incubated with LIF during different periods (ranging from 2 to 10 min). Phosphorylated JAK1 (Ph-JAK1) and constitutive JAK1 expression (JAK1) were assessed. ß-Actin was used as reference control. In JEG-3 cells, practically no bands are observed for JAK1, while a very slight signal is identified for Ph-JAK1, although its intensity seems not to vary with the period of treatment. These bands have been considered as unspecific signals. In JAR cells, LIF stimulation correlated with a fast and transitory increase of the phosphorylated form of JAK1 (see upper bands), while the signals of its non-phosphorylated form became slighter with longer periods of LIF stimulation (notice that the longer the period of LIF treatment, the slighter became the bands of non-phosphorylated JAK1). Taken these results, JAK1 has been hypothesized to play a role in the LIF signaling in JAR cells.

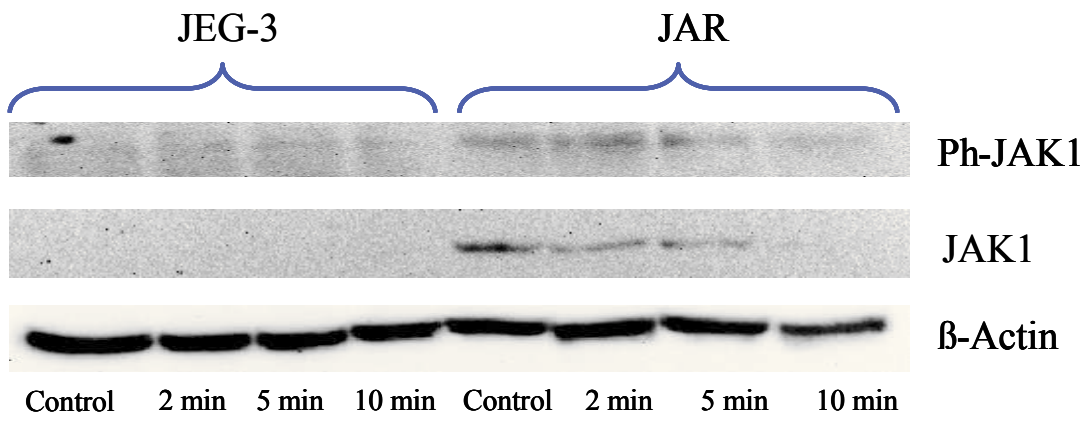


Fig. 19 Constitutive expression of JAK1 and its phosphorylation after LIF stimulation in JEG-3 and JAR cells (n=2)

Ph-JAK1: phosphorylated JAK1; JAK1: constitutively expressed JAK1.

Cell groups: Control (non-treated cells); 2 min, 5 min and 10 min (LIF-treated cells. Treatment length ranged from 2 min to 10 min).

Janus Kinase 2

The Western blot bands below (Fig. 20) derive from experiments in which JEG-3 and JAR cells were incubated with LIF during different periods (ranging from 2 to 10 min). Phosphorylated JAK2 (Ph-JAK2) and constitutive JAK2 expression (JAK2) were assessed. Lymphocytes stimulated with PHA (Lymph + PHA) were used as positive control for JAK2 expression. .ß-Actin was used as reference control. In JEG-3 cells, very slight signals were obtained using the JAK2 antibody, suggesting that this non-phosphoylated form is poorly expressed in these cells. Moreover, not very intense bands for phosphorylated JAK2 can be identified (with and without LIF stimulation). These bands do not vary significantly after LIF stimulation, suggesting that JAK2 is constitutively phosphorylated in these cells, although its concentration is not further affected by LIF. In JAR cells, no significant differences were observed in the bands of JAK2 after treating cells with LIF (bands from treated cells preserved basically the same intensity of control group). Moreover, LIF stimulation seems not to be involved in the phosphorylation of JAK2 in JAR cells, since practically no signal for Ph-JAK2 has been identified. These data support the hypotheses that JAK2 is not phosphorylated when JAR cells are stimulated with LIF (or maximally to a marginal degree), and might thus not be involved in the process of STAT3 phosphorylation triggered by LIF in these cells.

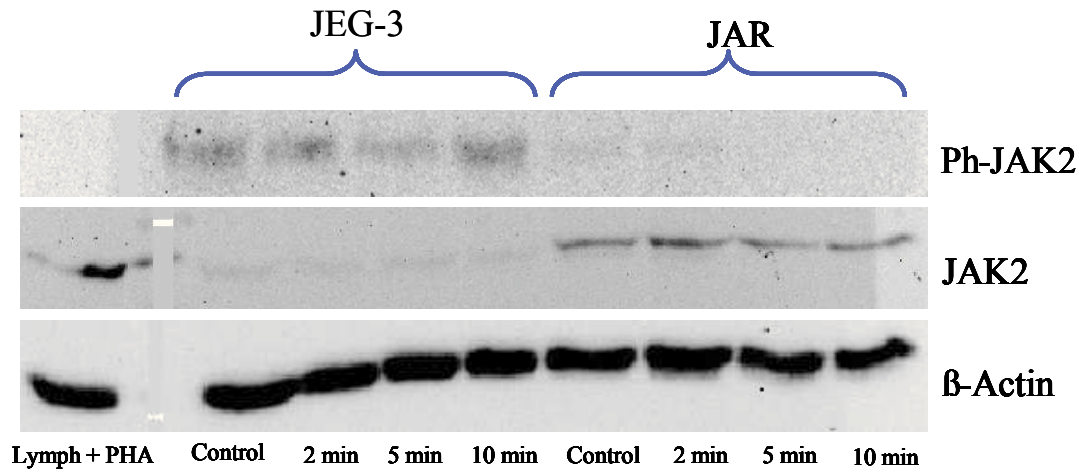


Fig. 20 Constitutive JAK2 expression and its phosphorylation after LIF stimulation in JEG-3 and JAR cells (n=2)

Cell groups: Lymph + PHA (lymphocytes stimulated with PHA); Control (non-treated cells); 2 min, 5 min and 10 min (LIF-treated cells. Treatment length ranged from 2 min to 10 min).

Janus Kinase 3

The Western blot bands below (Fig. 21) derive from experiments in which JEG-3 and JAR cells were incubated with LIF during different periods (ranging from 2 to 10 min). Phosphorylated JAK3 (Ph-JAK3) and constitutive JAK3 expression (JAK3) were assessed. Lymphocytes stimulated with PHA (Lymph + PHA) were used as positive control for JAK3 expression. .ß-Actin was used as reference control. The bands below evidence that neither non-phosphorylated nor phosphorylated JAK3 are expressed in JEG-3 and JAR cells (with or without LIF stimulation).

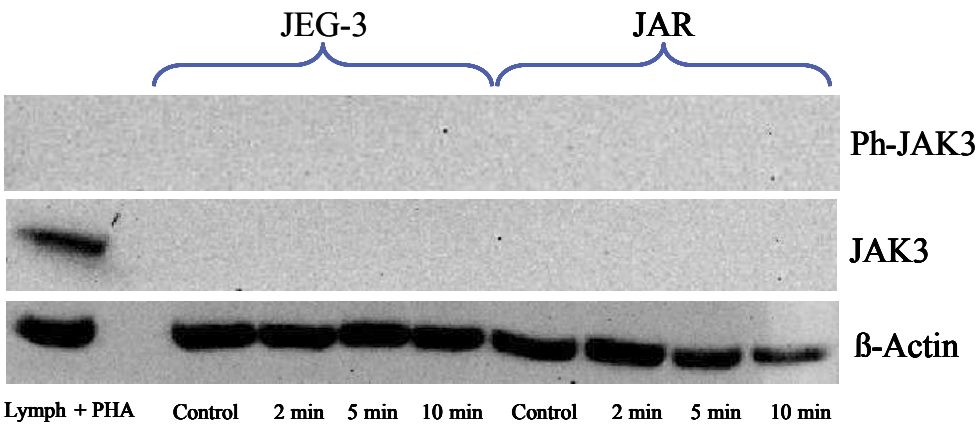


Fig. 21 Constitutive JAK3 expression and its phosphorylation after LIF stimulation in JEG-3 and JAR cells (n=2)

Ph-JAK3: phosphorylated JAK3; JAK3: constitutively expressed JAK3.

Cell groups: Lymph + PHA (lymphocytes stimulated with PHA); Control (non-treated cells); 2 min, 5 min and 10 min (LIF-treated cells. Treatment length ranged from 2 min to 10 min).

4.5 – JAK1 Knock-Down in JAR Cells

The previous results obtained by Western blot suggest that LIF is associated with the phosphorylation of Janus Kinase 1 (JAK1) in JAR cells. In order to test the hypotheses that a reduced expression of this isoform might impair the LIF-induced phosphorylation of STAT3, the technique of RNA interference was used to promote JAK1 knock-down in these cells.

To achieve an efficient gene silencing it is firstly necessary to establish an appropriate siRNA transfection method. Among the several possibilities, Nanofectin and Oligofectamine were

used in this work. Different concentrations of these transfection reagents and of the JAK1-specific siRNA sequence were tested, aiming to obtain the lowest gene expression.

Nanofectin

The Western blot bands below (Fig. 22.1) demonstrate that no effective JAK1 knock-down was reached in the experiments using Nanofectin for transfection. Different concentrations of the siRNA sequence (6 and 9 µl) and of Nanofectin (10 and 15 µl) were tested. The respective concentrations tested in each experiment are seen below in parentheses (after "siRNA" for the siRNA sequence and after "N" for Nanofectin). β-actin was used as control. Scrambled siRNA (seen as "Scr"), a siRNA with a scrambled non-genomic sequence, was used as control to verify possible unspecific effects elicited by transfection. Results are seen next. The intensity of the bands was quantified and values are seen in Fig. 22.2. The final value of each group comes from the ratio between JAK1 band and its respective β-actin counterpart. Values represent the arithmetic mean from both experiments, standardized with the values obtained in the non-genomic siRNA group (Scr / scrambled sequence), which was set as 100%. The values in Fig. 22.2 evidence that transfection with Nanofectin did not correlate with an efficient JAK1 knock-down in JAR cells, even testing different concentrations of this transfection reagent and of the siRNA sequence.

JAK1 Knock-Down Attempt Using Nanofectin

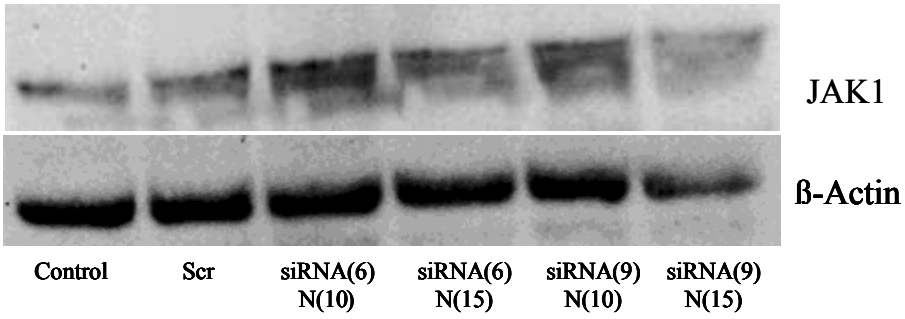


Fig. 22.1 JAK1 knock-down attempt using Nanofectin as transfection reagent (n=2)

JAK1: constitutively expressed JAK1.

Cell groups: Control (non-treated JAR cells); Scr (JAR cells treated with a scrambled non-genomic siRNA sequence); siRNA(x) N(y) (JAR cells incubated with different concentrations of JAK1-specific siRNA and Nanofectin. Respective concentration in parentheses).

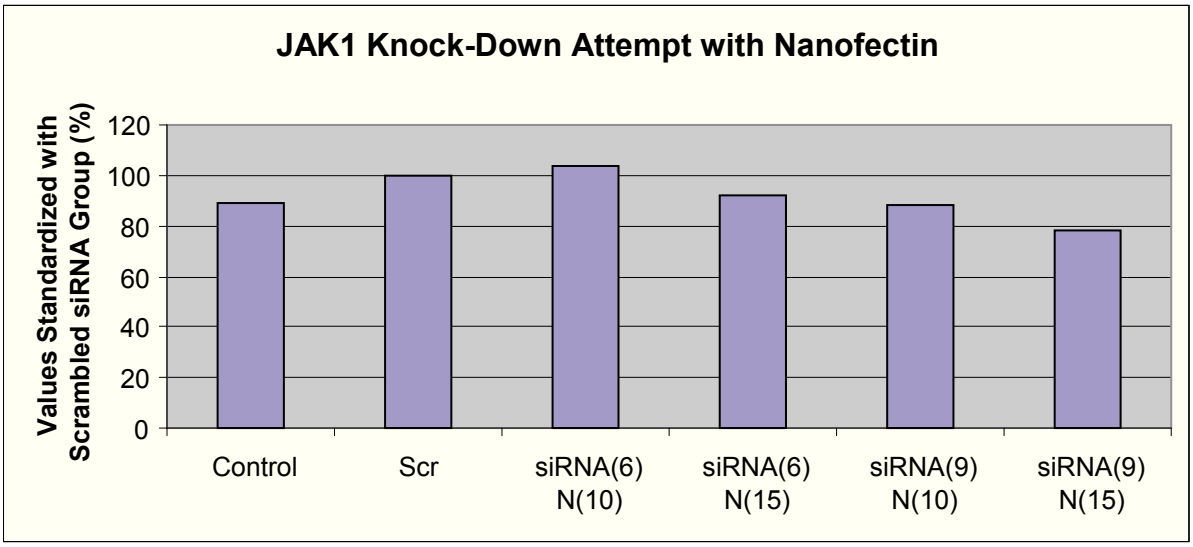


Fig. 22.2 JAK1 knock-down attempt using Nanofectin for transfection (quantification of Western blot bands)

Cell groups: Control (non-treated JAR cells); Scr (JAR cells treated with a scrambled non-genomic siRNA sequence); siRNA(x) N(y) (JAR cells incubated with different concentrations of JAK1-specific siRNA and Nanofectin. Respective concentrations in parentheses).

Oligofectamine

In the set of experiments using Oligofectamine, cells were incubated with this reagent and with the siRNA sequence for two different periods – 24 and 48 hours. Tested concentrations can be seen in Material and Methods. The main goal of the experiments was to obtain a reduced JAK1 expression. It was tested if a longer incubation time with the transfection reagent and the specific siRNA sequence might be related to a more effective gene knock-down. The experiment was repeated twice. The Western blot bands depicted in Fig. 23.1 show reduced JAK1 signals for the cells incubated with the siRNA sequence, both after 24 and 48 hours, indicating that relative gene suppression had been achieved. β-actin was used as control. Scrambled siRNA (seen as "Scr"), a siRNA with a non-genomic sequence, was used as control to verify possible unspecific effects related to transfection.

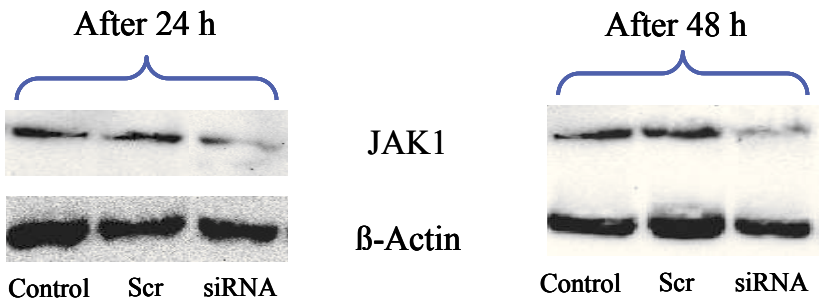


Fig. 23.1 JAK1 knock-down attempt using Oligofectamine for transfection (n=2)

JAK1: constitutively expressed JAK1.

Cells groups: Control (non-treated JAR cells); Scr (JAR cells treated with a scrambled non-genomic siRNA sequence); siRNA (JAR cells treated with a specific siRNA targeting JAK1). Oligofectamine was used as transfection reagent in Scr and siRNA groups. Cells were incubated with Oligofectamine and the specific siRNA during 24 or 48 hours.

In order to quantify these results, band intensity was measured and results are seen in Fig. 23.2. The values come from the quantification of Western blot bands. The experiment as a whole was performed twice. The values obtained by quantification of the bands of JAK1 (constitutively expressed JAK1) were divided by their respective β-actin counterpart. The values presented as next represent the arithmetic mean from both experiments, standardized with values obtained in scrambled non-genomic siRNA group (seen as "Scr"), which was set as 100%. The values indicate that relative gene suppression was achieved in the groups using the JAK1 siRNA sequence (seen in the figure as "siRNA" group). In the experiments testing 24 h incubation, JAK1 expression fell to approximately 65% (mean value, setting Scrambled as 100%). In the experiments testing 48 h incubation, this value was even lower (35%), suggesting that a longer incubation period with the specific siRNA sequence correlated with a more intense silencing. Considering these results, in all further experiments in which JAK1 siRNA was used, cells were incubated with this specific sequence for 48 h (assuming that gene suppression after this time was more effective than after 24 h).

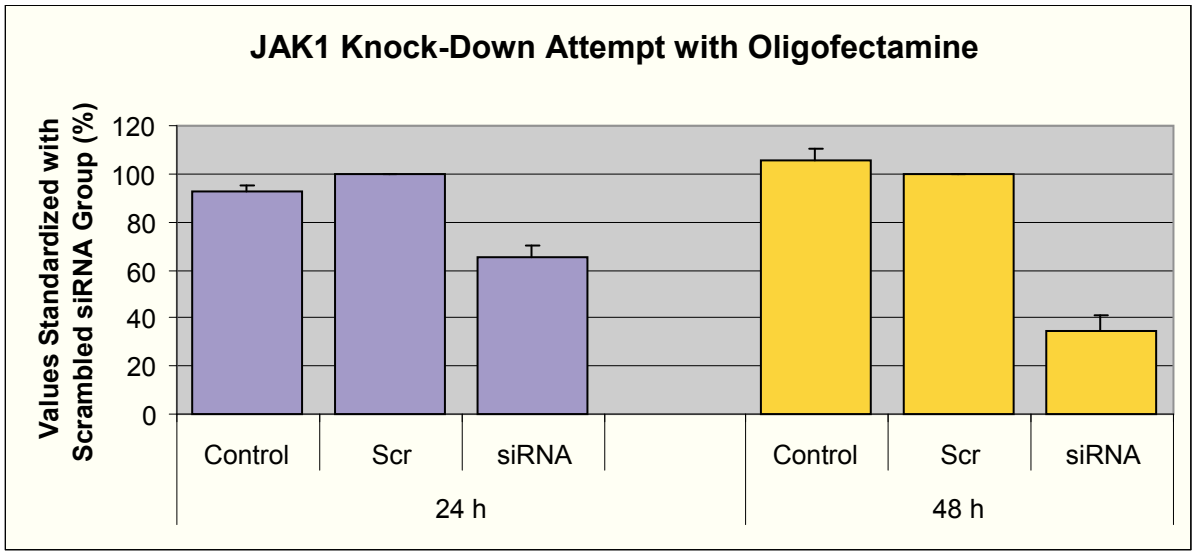


Fig. 23.2 JAK1 knock-down attempt using Oligofectamine for transfection (quantification of Western blot bands)

Cell groups: Control (non-treated cells); Scr (cells treated with a scrambled non-genomic siRNA sequence); siRNA (cells treated with a specific **siRNA** targeting JAK1).

Oligofectamine was used as transfection reagent in Scr and siRNA groups. Cells were incubated with Oligofectamine and the specific siRNA during 24 or 48 hours.

4.6 – JAK1 Knock-Down and the LIF-Induced Phosphorylation of STAT3 in JAR cells

In a further series of experiments, JAR cells were submitted to JAK1 knock-down (JAK1 KD) during 48 hours, using Oligofectamine for transfection. Cells were then incubated with LIF for different periods (2, 5 and 10 min). Activated tyrosine 705 STAT3 (Ph-STAT3) was assessed and β-actin was used as control. Scrambled siRNA (seen as "Scr"), a siRNA with a non-genomic sequence, was used as control to verify possible unspecific effects elicited by transfection. The Western blot bands depicted in Fig. 24 demonstrate that phosphorylated STAT3 is practically absent in the groups that had not been treated with LIF (see Control, Scrambled and JAK1 KD groups). Furthermore, LIF treatment was associated with STAT3 phosphorylation in JAK1-silenced cells. The longer the period of LIF incubation, the stronger became the signal of activated STAT3 (see JAK1 KD + LIF groups). These data reveal that JAK1 knock-down, at least in the silencing rates obtained in these experiments, was not enough to avoid STAT3 Tyr705 phosphorylation in these cells.

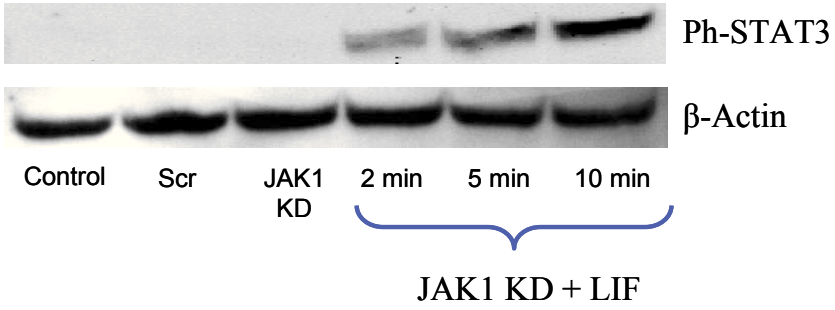


Fig. 24 Effect of JAK1 knock-down on the LIF-dependent STAT3 Phosphorylation in JAR cells (n=2)

Ph-STAT3: phosphorylated STAT3

Cell groups: Control (non-treated cells); Scr (cells treated with a scrambled non-genomic siRNA sequence); JAK1 KD (cells treated with the specific siRNA targeting JAK1); JAK1 KD + LIF (cells treated with the specific siRNA targeting JAK1, subsequently incubated with LIF during 2, 5 or 10 minutes).

Oligofectamine was used as transfection reagent in Scr and JAK1 KD groups.

4.7 – Proliferation, Migration and Invasion Assays

Functional experiments were performed to analyze possible effects of Leukemia Inhibitory Factor (LIF) on the proliferative, migratory and invasive behavior of JAR cells. Moreover, knock-down of JAK1 was performed to investigate the role of this JAK isoform on cell behavior. Considering that unspecific effects and changes on the functional parameters might be triggered by the transfection method itself and/or by the JAK1-specific siRNA sequence, cells transfected with a non-genomic sequence ("Scrambled siRNA") were taken as control in the experiments testing JAK1 knock-down. Finally, these cells (with reduced JAK1 expression) were stimulated with LIF to observe if this cytokine might modify cell behavior, even when a component of its signaling cascade had been disrupted. All experiments started with about 30,000 cells (aliquots were counted with a Neubauer chamber and cell number was extrapolated to total volume of culture). Cells were seeded into individual wells of a 24-well plate and were incubated during 24 hours. In those experiments in which LIF was used, cells were stimulated throughout the cultivation period at a final concentration of 10 ng/ml. Final

cell counting was performed by the flow cytometer. Results are shown as the final cell number (arithmetic mean, n=4 for each experiment).

Fig. 25 evidences the effects of LIF treatment on proliferation, migration and invasion of JAR cells. Cells were incubated with 10 ng/ml LIF during 24 hours and their behavior was compared to untreated cells (control groups). Data represent the arithmetic mean (values obtained using flow cytometry). Error bars show the standard error. Among the functional parameters assessed, LIF treatment was associated with a significant increase in cell proliferation rate ($p<0.05$, marked with a *). Differences on cell migration and invasion after treating cells with LIF were not significant.

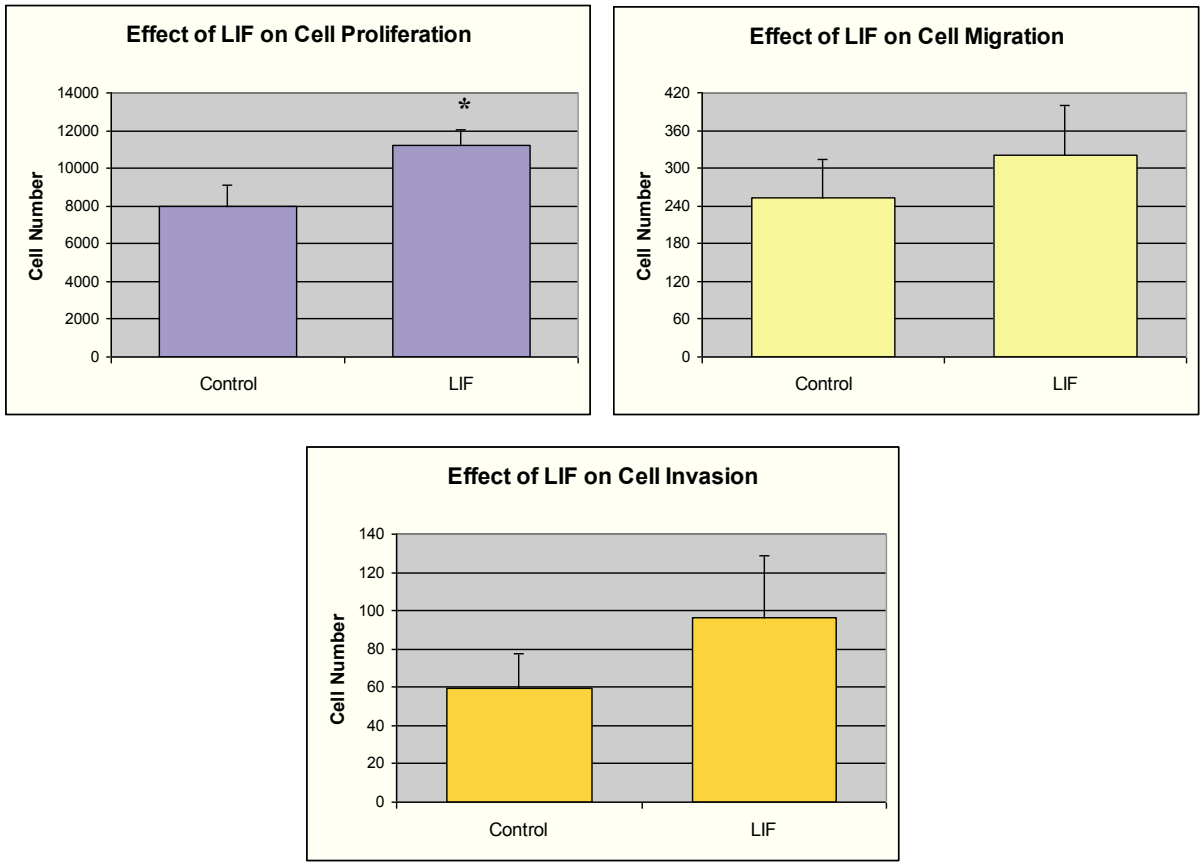


Fig. 25 Influence of LIF on the proliferative, migratory and invasive behavior of JAR cells (n=4)

Cell groups: Control (non-treated cells), LIF (cells incubated with LIF during 24 hours).

The figure below (Fig. 26) shows the effects of JAK1 knock-down (JAK1 KD) on the proliferation, migration and invasion of JAR cells. JAK1 KD groups were incubated with the specific JAK1 siRNA sequence for 48 hours. Oligofectamine was used for transfection. In the scrambled siRNA group (seen as "Scr"), cells were incubated for 48 hours with a non-genomic sequence using Oligofectamine for transfection. The scrambled group was used to verify possible unspecific effects triggered by RNAi. After a 48 hour transfection period, cells were submitted to functional tests, which lasted 24 hours. Data represent the arithmetic mean from experiments (values obtained using flow cytometry). Error bars show the standard error. The results show that JAK1 knock-down correlated with a significant decrease in cell migration rate ($p<0.05$, marked with a *, compared to Control and Scrambled groups). Differences on cell proliferation and invasion were not significant.

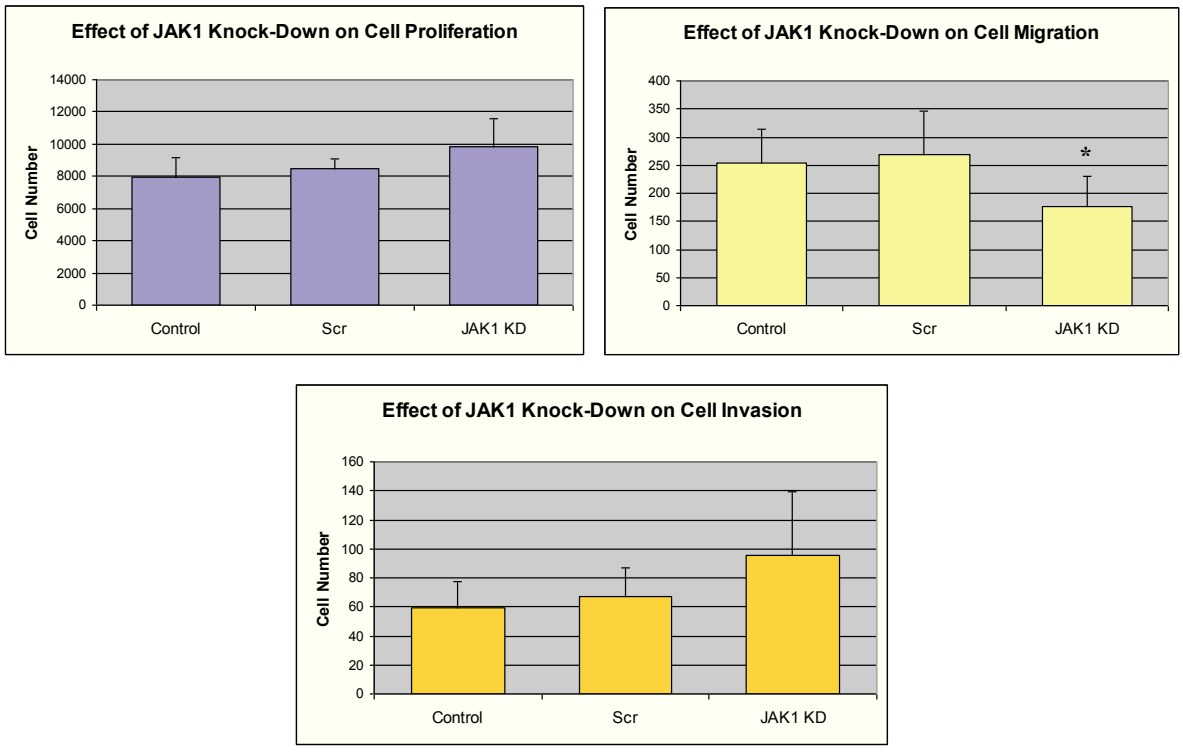


Fig. 26 Effect of JAK1 knock-down on the proliferative, migratory and invasive behavior of JAR cells (n=4)

Cell groups: Control (non-treated cells); Scr (cells treated with a scrambled non-genomic siRNA sequence); JAK1 KD (cells treated with the specific siRNA targeting JAK1).

The next figure (Fig. 27) demonstrates the effects of LIF on different functional parameters of JAK1-silenced cells (JAK1 KD). JAR cells were initially submitted to siRNA transfection using Oligofectamine. Cells were incubated with the specific JAK1 siRNA sequence for 48 hours. After this period, the subgroup JAK1 KD + LIF was treated with LIF for 24 hours. The subgroup JAK1 KD was also kept incubated for 24 hours, but without LIF stimulation. Data represent the arithmetic mean from experiments (values obtained using flow cytometry). Error bars show the standard error. The results below show that LIF treatment was associated with a significant increase in the migration rate of JAK1-silenced cells ($p<0.05$, marked with a *, compared to untreated silenced cells). No significant differences on cell proliferation and invasion were verified.

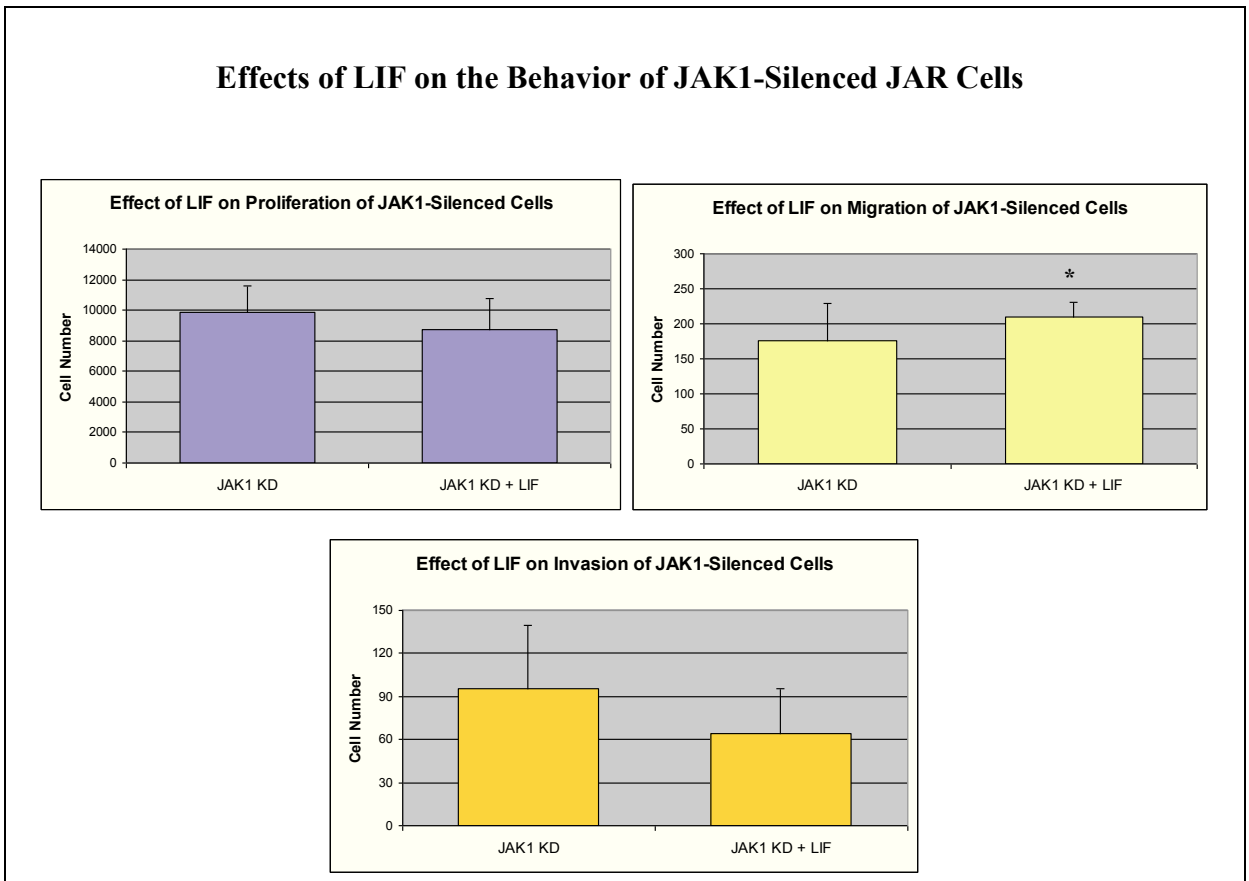


Fig. 27 Effect of LIF on the proliferative, migratory and invasive behavior of JAK1-silenced JAR cells (n=4)

Cell groups: JAK1 KD (cells treated with the specific siRNA targeting JAK1); JAK1 KD + LIF (cells treated with the specific siRNA targeting JAK1, subsequently incubated with LIF during 24 hours).

5. Discussion

5.1 – Influence of LIF and IL-6 on the STAT3 Tyrosine Phosphorylation in Different Choriocarcinoma Cell Lines

Cell Culture and Stimulation

In order to test the hypotheses that Leukemia Inhibitory Factor (LIF) and Interleukin-6 (IL-6) induce both the tyrosine (tyr705) phosphorylation of STAT3, different choriocarcinoma and hybrid cell lines were cultured separately and incubated with these factors at a final concentration of 10 ng/ml and 20 ng/ml respectively. Different periods of LIF and IL-6 stimulation were tested.

The way cells were incubated and stimulated must be critically analyzed. Fitzgerald et al. suggest that cells respond differently to the same factor according to cell physiology, utilized concentrations and stimulation periods (Fitzgerald et al. 2010). There is no consensus in literature regarding the optimal concentration of LIF and IL-6 for stimulation of trophoblast cells and choriocarcinoma cell lines *in vitro*. Primary trophoblast cells, for example, have been stimulated with LIF in concentrations ranging from 10 to 50 ng/ml in experiments investigating the effects of this cytokine on trophoblast differentiation (Nachtigall et al. 1996, Tapia et al. 2008). The lack of standardized values in *in vitro* experiments correlates with the difficulty in determining the physiological expression of these cytokines during the implantation phase. In parts, there are not standardized values because the real concentrations of these cytokines have not been completely established during pregnancy. For ethical reasons, it is not possible to assess directly the production of LIF by endometrial and trophoblast cells during human pregnancy. Furthermore, to date there is neither a validated tool to measure LIF expression in endometrial fluids, nor a consensus regarding threshold levels associated with implantation success (Brinsden et al. 2009). Beyond that, it should be highlighted that the effects of cytokines depend on optimal concentrations, and that disturbances in their fine regulation correlate with different pathologies. In these terms, both too high and too low levels of LIF expression have been identified in uterine flushings, endometrial cell cultures and decidual cells from women suffering from unexplained infertility or repeated abortions, suggesting that successful implantation depends on strictly regulated LIF levels (Ledee-Bataille et al. 2002, Delage et al. 1995, Piccinni et al. 1998).

Considering that a negative feed-back mechanism regulates the LIF signaling pathway, as previously described, it is expected that concentrations differing from the optimal one promote similar functional effects (Markert et al. 2011). Taking these data together, it is appropriate to emphasize the importance of establishing adequate threshold levels in experiments testing cytokines such as LIF and IL-6, whose effects strongly depend on their concentrations.

Considering the lack of data in literature concerning these optimal concentrations, we relied on the experience of our laboratory to counterbalance this technical limitation. A previous study conducted in our laboratory revealed, using immunoblot, that LIF stimulation evokes a dose and time-dependent phosphorylation of STAT3 in choriocarcinoma cells. In this study, strong signals of STAT3 activity have been detected after LIF stimulation (concentrations ranging from 10 to 100 ng/ml LIF) in all periods assessed (LIF treatment during 15, 30 and 60 min). In the same study, IL-6 elicited a very small degree of STAT3 tyrosine phosphorylation, detectable with 20 and 200 ng/ml IL-6, predominantly after a stimulation period of 30 min (Fitzgerald et al. 2005b). These results were used as reference in the present work, in which cells have been incubated with 10 ng/ml LIF or 20 ng/ml IL-6. Different effects should be expected in the groups of cells investigated in this work, since cell physiology depends on the specificities of cell metabolism and environment.

Influence of IL-6 and LIF on the STAT3 Tyrosine Phosphorylation

Several growth factors and cytokines are known to activate STAT3, including members of the IL-6 family (such as IL-6, IL-11 and LIF), IL-2 family (such as IL-2 and IL-15), epidermal growth factor (EGF) and hepatocyte growth factor (HGF) (Corvinus et al. 2003, Heinrich et al. 1998).

As demonstrated by Western blot, the results of this work show, indeed, that LIF is strongly associated with the tyrosine phosphorylation of STAT3 in different cell lines, including JEG-3, JAR, AC1-M59 and ACH-3P cells. The activation of STAT3 was observed even after a very short period of LIF stimulation (2 min) in JEG-3 and JAR cells. Similarly to previous studies, IL-6 elicited just a marginal degree of STAT3 activation in JEG-3, AC1-M59 and ACH-3P cells. Moreover, the results demonstrate that these choriocarcinoma and hybrid cell lines respond similarly as trophoblast cells, at least regarding the degree of STAT3 activation (Poehlmann et al. 2005).

In the context of tumor progression, STAT3 has been described as an inducer of malignant cell transformation, leading to cell longevity, hyperplasia and invasiveness (Epling-Burnette et al. 2001, Bromberg et al. 1999). Furthermore, it has been implicated in modulation of metastatic progression, as the STAT3-dependent transcription cascade has been demonstrated to induce angiogenic and inflammatory responses that facilitate tumor metastasis (Ranger et al. 2009).

As previously commented, LIF is assumed to facilitate embryo implantation and is expressed by human placenta and endometrium, particularly in the maternal-fetal interface at the time of implantation (Paiva et al. 2009, Vogiagis et al. 1996, Aghajanova et al. 2003, Bhatt et al. 1991). Different cell types, including ovarian stromal cells, hepatocytes and kidney epithelial cells are able to express LIF physiologically (Lass et al. 2001, Auernhammer and Melmed 2000). The expression of LIF by tumor cells themselves has been investigated over the last two decades, but studies are still rare and preliminary. Among these studies, LIF expression has been identified, using ELISA and/or immunohistochemistry, in human kidney, prostate and breast cancer cell lines (Dhingra et al. 1998, Kellokumpu-Lehtinen et al. 1996). In one of these studies, endogenously produced LIF correlated with elevated proliferation rates of kidney and prostate cancer cells (Kellokumpu-Lehtinen et al. 1996). Another study identified high levels of LIF expression in mouse mammary tumors growing *in vivo* and in their primary cultures, as analyzed by RT-PCR and immunohistochemistry, suggesting an autocrine/paracrine stimulation of STAT3 activation (Quaglino et al. 2007). To date, there are no definitive studies confirming LIF protein expression by trophoblastic tumors and their deriving hybrid cell lines. Considering the strong STAT3 phosphorylation elicited by LIF, it would be interesting to evaluate if such an autocrine stimulation mechanism exists in the cells tested in this work. This would contribute to better understanding the progression of trophoblastic tumors. Nonetheless, the indubitable STAT3 tyrosine phosphorylation elicited by LIF in trophoblastic and hybrid cell lines observed in this work reveals that these cells are at least strongly influenced by LIF, which, in turn, is believed to stimulate cell proliferation and induce tumor progression.

5.2 – Expression and LIF-Induced Phosphorylation of Janus Kinases in JEG-3 and JAR Cells

Once observed that LIF stimulation correlated with the tyrosine phosphorylation of STAT3 in JEG-3 and JAR cells, it was investigated which Janus Kinases (JAKs) might have been activated after LIF stimulation. In these terms, it was valid to scrutinize which JAK isoforms were constitutively expressed in these cells, and which of them might have been phosphorylated upon LIF binding to its receptor on cell surface.

As demonstrated by Western blot in this work, JAK1 and JAK2 are constitutively expressed in the choriocarcinoma cell lines JEG-3 and JAR. Practically no JAK3 signal has been identified in these cells. These results were reinforced by immunocytochemical studies, which evidenced similar patterns of expression, with a few differences. Furthermore, LIF stimulation correlated with the phosphorylation of JAK1, but not of JAK3 or JAK2 (in this case, if present, just to a very marginal degree). However, the limitations of the use of Western blot should be considered for these results, since the presence of small amounts of non-activated or activated forms might not have been detected.

The JAK/STAT pathway is usually referred to as the main signaling mechanism for a vast array of hormones, growth factors and cytokines (Rawlings et al. 2004). In mammals, four members of the JAK family have been identified, namely JAK1, JAK2, JAK3 and Tyrosine Kinase 2 (TYK2) (Yamaoka et al. 2004). Considering their intimate association with cytokine receptors, these isoforms are particularly observed in endosomes and cell membrane, where they get activated upon cytokine binding, as previously commented (Hofmann et al. 2004, Ragimbeau et al. 2003). Jak1, Jak2 and Tyk2 are ubiquitously expressed in mammals, while Jak3 is mostly expressed in hematopoietic cells (Kawamura et al. 1994, Musso et al. 1995, Tortolani et al. 1995). This data is congruous with the results of this work, in which Western blot bands for JAK1 and JAK2, but not for JAK3, have been identified in JEG-3 and JAR cells. Due to financial limitations, the expression of Tyk2 has not been evaluated in the present work. Additionally, seven STAT isoforms have been observed in mammalian cells (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6). All of them are ubiquitously expressed, except STAT4, which is restricted to thymus, myeloid cells and testis (Zhong et al. 1994, Costa-Pereira et al. 2011).

As previously described, several cytokines signal via JAKs, leading to phosphorylation of inner ligands, such as the STATs. These are known to translocate to the cell nucleus, where they induce the transcription of target genes (Yamaoka et al. 2004). The next table (Table 1)

shows some of the main cytokines which signal through specific members of the JAK family (Yamaoka et al. 2004).

Table 1 Cytokines signaling through the JAK family

JAK1	JAK2	JAK3	TYK2
Common γ chain family: (IL)-2, IL-4, IL-7, IL-9, IL-15, IL-21 Cytokines sharing gp130: IL-6, IL-11, oncostatin M, LIF, CNF, GM-CSF and IFNs	Hormone-like cytokines: GH, PRL, EPO, TPO Cytokines sharing IL-3 receptor: IL-3, IL-5 and GM-CSF Cytokines sharing gp130 Some IFNs	Common γ chain family: (IL)-2, IL-4, IL-7, IL-9, IL-15, IL-21	IL-12

IL (Interleukin), LIF (Leukemia Inhibitory Factor), CNF (Ciliary Neurotrophic Factor), GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor), IFN (Interferon), GH (Growth Hormone), PRL (Prolactin), EPO (Erythropoietin), TPO (Thrombopoietin).

Finally, the constitutive expression of these JAK isoforms has been assessed in villous and extravillous trophoblast cells collected from placental tissues of a healthy woman undergoing elective abortion in the 11th week of pregnancy. As it is known, villous trophoblasts are involved in gas and nutrient exchange between fetus and mother. They also have endocrine and endothelial functions. Extravillous trophoblasts are implicated in the anchoring of chorionic villi in the uterus, as long as they are able to migrate through the decidua and invade the spiral arteries, finally replacing and remodeling their walls (Tarrade et al. 2001). As demonstrated in the Results section, our immunocytochemical analysis identified strong signals of JAK1 and JAK2 in villous trophoblast cells, including the inner mononuclear layer (cytotrophoblast) and the outer multinucleated syncytiotrophoblast. Mostly observed in the cell cytoplasm, both JAKs were believed to be located in endosomes. Additionally, just a marginal degree of JAK expression has been identified in extravillous trophoblast cells. Practically no signal for JAK3 has been verified in both cell subtypes. The constitutive expression of JAKs by cells located in the fetal-maternal interface suggests that the JAK/STAT pathway is probably involved in the signaling of different factors present in this

microenvironment, some of them potentially involved in the fine regulation of placental development, ultimately assuring an adequate environment for embryo growth.

5.3 – Trying to Silence JAK1 in JAR Cells

Transfection and RNA Interference

After observing that JAK1 was constitutively expressed and activated by LIF in JAR cells, it was hypothesized that the suppression of JAK1 might disturb the LIF/JAK1/STAT3 phosphorylation cascade. In humans, it is known that mutations leading to constitutive activation or fail in regulation of JAK signaling lead to an array of pathological conditions, such as inflammatory diseases, erythrocytosis and leukemias (Rawlings et al. 2004). In this sense, it was investigated if the knock-down of JAK1 might impair STAT3 activation and modify cell behavior, altering functional parameters such as cell proliferation, migration and invasion.

In order to promote JAK1 knock-down and test these hypotheses, a set of experiments using RNA interference (RNAi) has been performed. As previously described, RNAi is a method to knock down gene expression post-transcriptionally, in which a specific mRNA is degraded after pairing with the complex RISC-siRNA. In this work it was used a synthetic siRNA that is cleaved intracellularly by the enzyme Dicer, originating a siRNA sequence targeted against JAK1. Aiming to establish an appropriate transfection method, two different lipid-mediated transfection reagents (Nanofectin and Oligofectamine) were tested. Furthermore, cells were submitted to different periods of incubation with the siRNA sequence (24h or 48h), in order to verify if treatment length could affect the effectiveness of silencing.

Transfection with Nanofectin and Oligofectamine

As previously shown in Results, no reproducible JAK1 knock-down was obtained in the set of experiments using Nanofectin as transfection reagent. Even testing different concentrations of Nanofectin and siRNA sequence, an insignificant silencing rate has been achieved. At this point, it must remembered that cell physiology, which substantially varies in different cell groups, may positively or negatively influence the activity of siRNAs. Moreover, a relatively high cellular death was observed in the experiments using Nanofectin. This could be explained by the fact that some transfection reagents (specially those with a liposomal

structure) induce the cellular release of inflammatory cytokines, such as interferon, which is able to block translation and to trigger sequence-nonspecific mRNA degradation, ultimately promoting the activation of cell apoptosis (Judge et al. 2005, Shinagawa and Ishii 2003). Furthermore, it has been suggested that cytoplasmic dsRNA and siRNA duplexes may activate the dsRNA-dependent protein kinase R (PKR) in nucleated cells. PKR has been related to cell growth inhibition through decreased protein synthesis and stimulation of interferon production (Saunders and Barber 2003, Geiss et al. 2001, Levy et al. 1969). In these terms, Nanofectin and/or PKR-induced production of interferon might have triggered apoptosis in these cells.

After observing that the experiments with Nanofectin had not been successful, a new set of attempts using Oligofectamine were carried out in order to promote JAK1 silencing. As demonstrated in Results, these experiments were operated in duplicate using different periods of incubation with the siRNA sequence, ultimately revealing better knock-down efficiencies, as demonstrated by Western blot. Setting the rate of JAK1 expression of the non-genomic sequence (Scrambled-RNA group) as 100%, the experiments using Oligofectamine revealed that the constitutive expression of JAK1 had fallen to about 65% after a 24h incubation with the siRNA sequence (or 35% knock-down efficiency) and to about 35% after a 48h incubation period (or 65% efficiency). Considering these results, the use of Oligofectamine and the incubation period of 48h with the siRNA sequence have been considered as standard for all subsequent experiments in which JAK1 knock-down was desired (for example, in the functional experiments).

Scrambled-siRNA as Control

In the set of experiments in which RNA interference (RNAi) was performed, the outcome of JAK1 silencing was assessed by Western blot. In order to quantify and compare knock-down efficiency, parallel samples were prepared using untreated cells and scrambled-siRNA (Scr-siRNA). The latter is composed of a non-genomic siRNA sequence (i.e. it has no match with any known human mRNA) which is transfected to verify the specificity of the designed siRNA and test the occurrence of unspecific effects possibly triggered by the transfection method on the targeted protein. In this work, Scr-siRNA groups were considered as control and the results stemming from samples cultured with the Scr-siRNA demonstrated relatively few changes on JAK1 expression and on cell behavior profiles when compared with untreated cells. However, it should not be discarded that even this non-genomic sequence might trigger

unspecific effects on cells, including both up and down-regulation of untargeted genes. In these terms, only a global gene expression study might have elucidated the effects of the Scr-siRNA sequence in these cells.

JAK1 Knock-Down in JAR Cells

With the use of Oligofectamine and a JAK1-specific siRNA sequence, the constitutive expression of JAK1 in JAR cells have been reduced to about 35%, as demonstrated by Western blot. In order to test the hypothesis that such degree of silencing might have influenced the rates of LIF-induced STAT3 phosphorylation, these cells have been treated with LIF for different periods of time and the rates of STAT3 phosphorylation have been assessed by Western blot. As observed in Results, LIF treatment continued to be associated with STAT3 Tyr705 phosphorylation in the JAK1-silenced groups. The longer cells were incubated with LIF, the higher were the rates of STAT3 phosphorylation.

At this point it must be considered that the 65% reduction of JAK1 expression might have not been high enough to prevent the activation of STAT3. More experiments are thus necessary to reach better knock-down rates. In these terms, it would be interesting to test different siRNA sequences targeting JAK1. Generically, the use of a pool of siRNAs with the same target is intended to reduce the occurrence of sequence-dependent off-target effects. Additionally, it should be considered that not all siRNAs targeting a common sequence demonstrate the same knock-down efficiency, so that the use of different siRNAs might equilibrate this disbalance (Sledz and Williams 2005).

It is also plausible that STAT3 might have been activated by mechanisms of crosstalk between LIF, JAKs and other pathways. A vast range of pathway intercommunication has been described over the last years, such as the cooperation between the JAK/STAT and Notch pathways leading to STAT3 phosphorylation (Kamakura et al. 2004). In these terms, LIF might have induced STAT3 activation by means other than the regular LIF/JAK/STAT pathway.

Although stimulation with LIF has correlated with practically no phosphorylation of JAK2 in JEG-3 and JAR cells in this work (or maximally to a very marginal degree), it should be remembered that IL-6 type cytokines have been reported to activate JAK1, JAK2 and TYK2, leading to STAT3 phosphorylation (Heinrich et al. 1998). Considering that TYK2 expression and activation has not been assessed in this work, it is quite possible that part of the activated

STAT3 identified by Western blot had been expressed through the LIF-induced JAK2 and TYK2 phosphorylation.

siRNA Limitations and Possible Alternatives

An efficient gene knock-down by means of RNA interference (RNAi) depends on several factors. Over the last years different techniques have been developed in an attempt to upgrade gene silencing efficiency and to bypass common limitations, ranging from optimization of the siRNA design and of transfection methods to avoidance of off-target effects (Harborth et al. 2003). Designing adequate silencing triggers (considering both siRNA sequence and structure), as well as assuring efficient siRNA uptake into the cell by the choice of an appropriate transfection method are usually considered crucial steps for an efficient gene knock-down (Siolas et al. 2005).

Cells respond differently to RNAi, and some of them are considered hard-to-transfect, like adipocytes and some fibroblast, epithelial and neuronal cell lines (Lab Times datasheet 2010). Furthermore, siRNA uptake varies considerably among cells (Kim et al. 2005), as well as the concentration of available RISC (Harborth et al. 2003, Gonczy et al. 2000). Further factors influencing silencing efficiency include cellular proliferation rate and half-life of target message and/or protein (Sledz and Williams 2005).

Although siRNAs are relatively stable when conditioned in cell culture (Reinhart et al. 2000), target silencing activity is usually transient in mammalian systems and persists for approximately 3-7 days (Elbashir et al. 2002, Holen et al. 2002, Yang et al. 2001). This is valid especially for proliferating cells, and a conceivable reduction of the number of RISCs carrying siRNA upon cell division (basically a “siRNA dilution”) has been suggested to explain this transient effect (Omi et al. 2004).

Considering the difficulty in achieving a stable gene silencing in many cells, some alternatives for siRNA have been developed. One of them is based on the use of short-hairpin RNA (shRNA), whose name derives from the tight hairpin turn present in its structure. shRNA technology uses plasmid or viral vectors coding specific sequences that get stably integrated into the genome (Czauderna et al. 2003). The transcription of these sequences by polymerase III originates shRNAs, which are then processed into siRNAs in the cytoplasm. Some vectors have been designed to induce themselves the expression of polymerase III, ensuring the shRNA to be constantly expressed and even to be passed through the germline (Matsukura et al. 2003, Sijen et al. 2001). The originated siRNAs are subsequently bound to

RISC, the complex that will bind and cleave targeted mRNAs, ultimately promoting post-transcriptional gene silencing (McIntyre and Fanning 2006). The stable integration of the vectors into the genome is responsible for a long-term silencing of targeted transcripts *in vitro* and *in vivo* (Dorsett and Tuschl 2004), turning shRNA a promising tool for RNA interference.

5.4 – Cell Behavior After LIF Stimulation and JAK1 Knock-Down

Proliferation

As previously discussed, LIF treatment correlated with an intense tyrosine phosphorylation of STAT3 in JEG-3, JAR, AC1-M59 and ACH-3P cells, as demonstrated by Western blot. Additionally, JAR cells that had been incubated with LIF exhibited higher proliferation rates than untreated cells, as demonstrated by cell counting performed by the flow cytometer. Interestingly, these results are similar to a previous study from our laboratory, in which the proliferation rates of JEG-3 cells treated with LIF have also been found elevated (Fitzgerald et al. 2005b).

Taken together, this data suggests that the LIF/JAK/STAT3 signaling pathway is implicated in regulation of cell proliferation in JAR cells. As it is known, different malignancies have been reported to express phosphorylated STAT3 constitutively. This aberrant activity has been associated to up-regulation of cell cycle progression and inhibition of apoptosis, leading cells to exhibit a malignant behavior (Bromberg et al. 1999, Epling-Burnette et al. 2001). Additionally, LIF and STAT3 have been described as promoters of proliferation in certain cell types, including neuronal stem cells, human erythroid progenitor cells, trophoblast and JEG-3 cells (Oshima et al. 2007, Ratajczak et al. 1997, Nakashima et al. 1999, Fitzgerald et al. 2005b). In rats, the peak of maternal LIF at GD14.5 has been recently demonstrated to be followed by an increase of LIF concentration in the fetal serum and fetal cerebral spinal fluid, culminating in the proliferation of neuronal progenitor cells in the cerebrum of the fetuses (Simamura et al. 2010). Although not confirmed, a temporary elevation of LIF levels in human maternal serum has been suggested to play a similar role on human cerebral histogeneses (Pitard et al. 1998). In this sense, LIF seems to play a role in the stimulation of cell proliferation in the physiological and pathological settings.

Migration and Invasion

The process of invasion embraces adhesion, migration and invasion of a cell into a determined tissue. Many similarities have been described between trophoblast and tumor cells regarding their capability of growing invasively and escaping from damaging responses triggered by the immune system (Murray and Lessey 1999). Malignant tumor cells abuse these abilities and grow in an uncontrolled fashion, while trophoblast cells, inversely, invade in a strictly time and space controlled manner (Bischof and Campana 2000, Murray and Lessey 1999). The process of invasion is locally regulated by several factors, including hormones, growth factors and cytokines, such as LIF. The interactions between trophoblast cells themselves, trophoblast/extra-cellular matrix components and trophoblast/immune cells depend on a multitude of extra and intracellular signals (Fitzgerald et al. 2010).

Maximally observed in the maternal-fetal interface at the time of implantation, LIF has been implicated in regulation of cell invasiveness, although reports about its effects on different cells types have been controversial (Aghajanova et al. 2003, Vogiagis et al. 1996, Bhatt et al. 1991). As previously resumed in this work, LIF has been shown to stimulate the production of TIMP-1 and -2 by human extravillous trophoblast cells, which act as counterparts of matrix metalloproteinases (MMPs) (Tapia et al. 2008). As it is known, MMPs enable trophoblast cells to invade maternal decidua upon extra-cellular matrix degradation. Moreover, LIF has been shown to induce decreased gelatinase activity in first trimester trophoblast cells (Nachtigall et al. 1996, Bischof et al. 1995). Considering that LIF correlates with decreased gelatinase activity, increased TIMP production and increased expression of adhesion molecules by trophoblasts, it may be concluded that this cytokine exerts an overall inhibitory effect on trophoblast invasion (Nachtigall et al. 1996, Bischof et al. 1995).

In this work, neither the incubation of cells with LIF, nor the disruption of the LIF signaling pathway by RNA interference have increased or decreased cell invasiveness significantly. At least to JAR cells, LIF stimulation and JAK1 silencing seem not to modify this functional parameter. It is possible that LIF exerts an inhibitory effect on the invasive behavior of JAR cells, similarly as in trophoblasts. On the other hand, a positive influence of LIF on the invasive behavior of JEG-3 cells has been identified using a similar matrigel assay in a previous work from our laboratory (Poehlmann et al. 2005). These conflicting results suggest that cellular response to LIF may vary among different choriocarcinoma cell lines. Along with these results, the effect of LIF on cell invasiveness has been demonstrated to vary even in subsets of the same cell sort. For example, the secretion of MMP-9 by laminin receptor $\alpha_6\beta_4$

positive cytotrophoblast cells has been demonstrated to be decreased after LIF treatment (thus suggesting that LIF inhibits invasion in these cells), whereas an unaltered production of MMP-9 has been identified in another cytotrophoblast subset (namely in fibronectin receptor $\alpha_5\beta_1$ positive cytotrophoblast cells) (Bischof et al. 1995). In contrast, elevated MMP-9 production by mouse blastocysts and human trophoblasts has been identified following LIF treatment (Harvey et al. 1995, Sunder and Lenton 2000), suggesting that LIF stimulates invasion in these cells. On the other hand, another report showed no significant differences in MMP secretion by human extravillous trophoblast cells following LIF treatment (Tapia et al. 2008).

At this point it must be considered that a balance between MMPs and TIMPs has been implicated in modulation of trophoblast invasion, and that normal placentation might be the result of this balance (Seval et al. 2004). In these terms, increased levels of gelatinases A and B have been identified in human first trimester intermediate trophoblasts (moving the aforementioned balance towards invasiveness), whereas increased levels of TIMPS prevailed at term (thus attenuating signals that induce invasion) (Polette et al. 1994). Furthermore, the specific role of distinct MMPs in the progression of malignancies has not been completely clarified, and some reports evidenced unexpected events triggered by them. For example, several MMPs (such as MMP-3, -7, -9 and -12) have been demonstrated to generate angiostatin from plasminogen, what could in turn limit angiogeneses in peritumoral areas and thereby inhibit tumor growth and invasion (Westermarck and Kahari 1999). These results go against the simplified hypothesis that the production of MMPs necessarily correlates with induction of cell invasiveness. Although not assessed in the present work, the levels of MMPs might have been counterbalanced by TIMPs after treating JAR cells with LIF. This could in turn justify the apparent irresponsiveness of these cells regarding their invasive behavior.

Taken together, these data suggest that LIF evokes different responses among cells and that its role still needs to be clarified. In this sense, a standard cell response following LIF treatment should not be expected. As previously described, JAR cells have been incubated during 24 h with this cytokine in this work. Although no significant effect on cell migration has been identified after this treatment, JAK1 gene suppression (induced by specific RNA interference) correlated with a significant decrease in cell migration rates ($p<0.05$). This suggests, at least to a certain point, that the JAK/STAT signaling pathway might play a role in the process of cell migration, independently from LIF. After treating JAK1 silenced cells with this cytokine, a significant increase in cell migration was observed in comparison to silenced untreated cells ($p<0.05$), suggesting that silenced cells do respond to LIF. Interestingly, these

results were not statistically significant when compared to non-silenced treated cells. If a 24h incubation period with LIF did not influence the migratory behavior of non-silenced cells, it could be equally expected no differences regarding this parameter to be identified in JAK1 silenced cells. But JAK1 silenced cells treated with LIF responded differently from JAK1 silenced untreated cells, suggesting that knock-down, rather than LIF, might have modified the migratory profile of these cells. This might be an off-target effect of the RNA interference method utilized in this work.

Finally, it should be considered that many reports investigating the role of LIF on migration and proliferation use different concentrations and stimulation periods, since no standard protocols can be extrapolated to distinct cell types. Furthermore, it must be remembered that each cell line presents differences in cell physiology, and that an expected response to a certain cytokine or growth factor may be influenced by a multitude of other factors. In this sense, discrepancies can be expected in studies investigating placentation and tumor progression.

5.5 – The JAK/STAT Signaling Pathway and Pregnancy

As it is known, adequate fetal anchorage and placentation demand strict control of cell proliferation, migration and invasion, which is in part regulated by a multitude of factors signaling inter and intracellularly (Tapia et al. 2008, Genbacev et al. 1992). Although many advances in the field of reproductive biology have been reached over the last decades, the role of many factors governing embryo implantation is still far from being completely understood. Even the signaling pathways of some cytokines still have to be clarified, and difficulty raises as crosstalks among them have been described (Fitzgerald et al. 2008). Studies in this area are special focus of interest, since dysregulation of the underlying mechanisms governing embryo implantation has been associated to adverse pregnancy outcome and infertility (Dimitriadis et al. 2010).

In this sense, dysregulation of LIF and its underlying JAK/STAT signaling pathway has been repeatedly associated to impairment of human and murine pregnancies (Dimitriadis and Menkhorst 2011). In murine models, for instance, disturbances in the LIF/JAK/STAT pathway have been related to poor pregnancy outcome. *Lif*-deficient mice are infertile because their blastocysts are unable to implant, although this situation can be reversed by infusion of LIF into the uterus (Stewart et al. 1992). Furthermore, *Stat3* knock-out embryos degenerate

and die shortly after implantation (Takeda et al. 1997), while *Socs3* knock-out embryos present impaired placental development, with reduced formation of spongiotrophoblast, labyrinthine layers and increased trophoblast giant cell differentiation (Fitzgerald et al. 2009). Specific deletions in the LIF underlying pathway have been tested in mice over the last decades in an attempt to understand the role of its factors, and the outcome of these experiments has been summarized in Table 2:

Table 2 Outcome of specific deletions in the LIF/JAK/STAT signaling pathway in mice

***Lif*-deficient mice** (females lacking functional LIF gene)	Failure of blastocyst implantation, although blastocysts are viable (Stewart et al. 1992)
***Lifr*-deficient mice**	Impaired placenta function, severe osteopenia, death within 24 h after birth (Ware et al. 1995)
***gp130*-mutant mice** (with deletion of all STAT-binding sites)	Failure of blastocyst implantation, impaired humoral and mucosal immune and hepatic acute phase responses (Ernst et al. 2001)
***Jak1*-deficient mice**	40% lower weight, defective lymphoid development and function (with severe combined immunodeficiency - SCID), perinatal death due to neurological deficits (with deficient suckling) (Rodig et al. 1998, Yamaoka et al. 2004)
***Jak2*-deficient mice**	Embryo death due to disrupted erythropoiesis. Normal placenta (Neubauer et al. 1998, Parganas et al. 1998)
***Jak3*-deficient mice**	Severe combined immunodeficiency (SCID), with defects in B lymphocyte maturation an T lymphocyte activation (Park et al. 1995, Thomis et al. 1995)
***Tyk2*-deficient mice**	Susceptibility to parasite infection, due to defective response to lipopolysaccharide (found in the outer membrane of Gram-negative bacteria) (Karaghiosoff et al. 2003)

Such studies find an obvious resistance in humans due to ethical reasons. The role of many factors expressed in pregnancy and their underlying pathways are then hypothesized to be similar in humans. Consequently, such extrapolations must be critically analyzed.

Anyway, disturbances in the LIF/JAK/STAT pathway have also been related to poor pregnancy outcome in humans. For example, decreased production of LIF mRNA by decidual leukocytes correlates with unexplained recurrent abortion (Piccinni et al. 2000). Furthermore, abnormal persistence of the LIF receptor on extravillous trophoblast cells and decreased SOCS3 expression in the villous tissues have been identified in placental beds from women suffering of early-onset pre-eclampsia (Fitzgerald et al. 2010, Reister et al. 2006). The placenta of women who develop this condition is characterized by poor decidual invasion and excessive proliferation of immature trophoblast (Reister et al. 2006, Redline and Patterson 1995).The importance of this data lye in the fact that pre-eclampsia is considered the major cause of maternal morbidity in antenatal care in developed countries, and women who have presented this condition are at increased risk of cardiovascular diseases later in life (Redman and Sargent 2005, Smith et al. 2001). Moreover, some women with pre-eclampsia also develop intrauterine growth restriction, a condition associated with impairment of maternal spiral arteries remodeling (Fisher 2004). As it is known, intrauterine growth restriction triggers perinatal mortality and the babies who have suffered from it are at an elevated risk of developing diabetes and cardiovascular complications in adult life (Solomon and Seely 2004). Taken together, this data highlights the importance of LIF and the JAK/STAT signaling pathway for a successful early pregnancy. They also justify the efforts in understanding the biological mechanisms governing embryo implantation and development, since disturbances in these pathways partly account for some of the most important disorders related to pregnancy.

5.6 – The JAK/STAT Signaling Pathway and Cancer

Over the last years, it has been investigated the components of abnormal microenvironments that play a role in the development of tumors, supporting the proliferation and invasive behavior of malignant cells (Sanz-Moreno et al. 2011, De Wever and Mareel 2003). These microenvironments are constituted of extracellular components, as well as tumor, stromal, inflammatory and immune cells (Mantovani et al. 2008). The regulation of these microenvironments is in part orchestrated through the expression of a multitude of factors, including chemokines, cytokines and their receptors (such as those of the IL-6 family), as well as by proteases and growth, survival and angiogenic factors (DeNardo et al. 2008, Mantovani et al. 2008, Bromberg and Wang 2009, Coussens and Werb 2002).

Several cytokines present in these microenvironments have been reported to signal through the JAK/STAT signaling pathway, in which the activation of JAKs leads to the phosphorylation of members of the STAT family, among other substrates, resulting in gene expression (Pedranzini et al. 2004, Yu et al. 2009).

Considered as key components for tumor progression, some cytokines are implicated, among others, in extracellular matrix remodeling, a process that involves integrins, metalloproteinases and force generation (Meshel et al. 2005, Gaggioli et al. 2007, Provenzano et al. 2006). The importance of actomyosin contractility, for instance, is evidenced by its role in the process of tumor cell movement. Carcinoma-associated fibroblasts use this contractile force, along with their proteolytic activity, to create tracks for tumor migration, enabling tumor cells to move as individual cells or in a collective fashion (Gaggioli et al. 2007, Sanz-Moreno et al. 2011). Furthermore, actomyosin contractility is believed to provide malignant cells the mechanical strength necessary to resist to the shear forces of the circulatory system during the process of metastasis (Sanz-Moreno et al. 2008, Gaggioli et al. 2007, Pinner and Sahai 2008). This force generated by actomyosin contraction has been shown to depend on cytokines, such as LIF, signaling through the receptor subunit gp130-IL6ST and the kinases JAK1 and Rho-Rho (ROCK) (Sanz-Moreno et al. 2011). At the same time, cytokines induce recruitment of inflammatory cells, leading to expression of proteases, proinvasive factors and more cytokines (Coussens and Werb 2002).

As previously commented, dysregulation of the JAK/STAT signaling network has been implicated in a multitude of pathological conditions. Aberrant JAK activation, for example, has been associated with the progression of leukemias and inflammatory disease (Rawlings et al. 2004, Yamaoka et al. 2004). Although not completely understood, dysregulated cytokine signaling has been suggested to be the most common mechanism leading to aberrant JAK activation, and this mechanism has been identified in hematologic malignancies, as well as in prostate and breast cancer (Pedranzini et al. 2006). Similarly, aberrant STAT3 activity has been implicated in cell cycle progression and apoptosis inhibition, inducing cells to develop a malignant behavior (which embraces hyperplasia, longevity and invasiveness) (Epling-Burnette et al. 2001, Bromberg et al. 1999). STAT3 has also been demonstrated to regulate the expression of proteinases involved in tumor invasion, such as matrix metalloproteinases MMP-1, MMP-2, MMP-9 (Song et al. 2008, Itoh et al. 2006). Additionally, aberrant activation of STAT3 correlates to the overexpression of mucin-1, which has important roles in tumorigenesis, mediating cancer cell survival and metastasis (Gao et al. 2009). Indeed, STAT3 can be considered a mediator of oncogenesis, and its constitutive activation has been

reported in a variety of malignancies, including brain, breast, lung, prostate, melanoma and squamous cell carcinomas (Bromberg et al. 1999, Catlett-Falcone et al. 1999, Pedranzini et al. 2004). As demonstrated in this work, LIF correlated with an intense phosphorylation of STAT3 in different choriocarcinoma cell lines. It is so plausible to hypothesize that an aberrant JAK/STAT3 activation in these cells might be triggered, at least in parts, by this cytokine, leading to cells to develop and perpetuate a malignant behavior.

5.7 – Conclusion and Perspectives

Conclusion

This work intended to clarify the function and regulation of some components of the JAK/STAT pathway involved in the signaling of LIF in different choriocarcinoma cell lines. As previously commented, a set of experiments was performed in order to analyze the influence of this cytokine on the STAT3 phosphorylation and its underlying mechanisms, such as the activation of some members of the Janus Kinase family. Therefore, it has been investigated which members of this family were constitutively expressed and activated upon LIF treatment in these cells. The presence of these JAK members in human placental tissues was additionally investigated by immunocytochemical tests. By applying functional assays, the effects of LIF on cell behavior have been investigated. Furthermore, the technique of RNA interference was used trying to establish an efficient JAK knock-down. Finally, the effects of this gene silencing on cell behavior and on possible disturbances in the JAK/STAT signaling pathway have been scrutinized.

Taken together, the results of this work demonstrate that:

- LIF (but not IL-6) induces an intense STAT3 phosphorylation in the tested choriocarcinoma cell lines;
- JAK1 and JAK2 are constitutively expressed in JAR cells, but just to a marginal degree in JEG-3 cells;
- Human villous syncytio and cytotrophoblast cells express JAK1 and JAK2;
- LIF treatment induces JAK1 phosphorylation in JAR cells and JAK2 activation in JEG-3 cells;
- LIF treatment correlates with a significant increase in the proliferation rate of JAR cells, but does not significantly influence their migratory and invasive behavior;

- JAK1 knock-down was more efficiently achieved by using Oligofectamine than Nanofectin as transfection reagent;
- LIF treatment induces STAT3 phosphorylation in JAK1-silenced cells, suggesting that other components beyond JAK1 may be involved in the signaling of this cytokine in JAR cells;
- JAK1-silenced cells respond differently from non-silenced cells. After LIF treatment, JAK-1 silenced cells demonstrated higher migration rates. On the other side, proliferation and invasion rates maintained unaltered.

Perspectives

Over the last years, the role of cytokine signaling in regulation of tumor cell dissemination has been investigated. A multitude of factors, including high levels of cytokines, are found within tumor microenvironments, and these factors are partially produced and regulated by cancer cells themselves or by tumor-associated cells, such as fibroblasts and inflammatory cells (Melnikova and Bar-Eli 2009, Chen et al. 2008). Some of the cytokines mediating cancer progression have been shown to signal through common specific pathways, such as the JAK/STAT signaling pathway. Key factors of this network have been demonstrated to be similarly expressed in a variety of tumors, such as constitutively activated STAT3, which has been identified, as previously cited, in brain and lung carcinoma, melanoma, choriocarcinoma etc (Fitzgerald et al. 2005b, Bromberg et al. 1999, Pedranzini et al. 2004).

The JAK/STAT signaling pathway has been recently suggested to provide the basis for sustained responses necessary for tumor invasion, at least in human melanoma (Sanz-Moreno et al. 2011). In these terms, a positive feedback resulting in enhanced STAT3 phosphorylation has been identified, with gp130-IL6ST, JAK1 and ROCK exerting central roles in the signaling network modulating the motility of tumor cells. Although the STAT3-dependent process of tumor development has not been completely clarified, aberrant STAT3 has been implicated in tumor growth and survival, angiogenesis, immunological masking, invasion and metastasis (Turkson 2004, Haura et al. 2005, Yue and Turkson 2009).

Reasonably, the comprehension of the role of the several factors involved in cancer initiation and progression can provide the bases for the development of therapeutic agents targeting its key components and pathways, including aberrant signaling transduction, what can potentially result in inhibition of tumor invasion and metastasis. As a matter of fact, therapeutic agents intended to block cancer progression have been developed over the last years. Among them,

tetracyclic pyridone 6 (P6) is a pan-JAK inhibitor which has been demonstrated to promote growth arrest and apoptosis of primary myeloma cells and myeloma-derived cell lines, both in culture and grown on bone marrow-derived stromal cells (Pedranzini et al. 2006). Additionally, tyrphostin AG490 is a JAK2 inhibitor which has been shown to restrain the growth of tumor cells *in vitro* and *in vivo* in acute lymphoblastic leukemia (Meydan et al. 1996, Levitzki 1996), highlighting the therapeutic potential of JAK blockade in the treatment of some tumors. Nevertheless, there is some concern about the amount of inhibitor necessary for this effect on tumor growth and the specificity of these factors, particularly of AG490 (Thompson 2005, O'Shea et al. 2005).

Aberrant STAT3 expression has also been targeted in molecular therapy, and STAT3 inhibitors have been developed since 2001 (Turkson et al. 2001). The most explored strategies targeting the STAT3 signaling pathway have been recently reviewed (Yue and Turkson 2009) and comprise:

- direct targeting of STAT3: dimerization inhibitors, DNA binding inhibitors and N-terminal domain inhibitors;
- indirect targeting of its intrinsic upstream components.

Much of the efforts in the field of STAT3 biological inhibition have been directed at disruption of dimerization. Although many STAT3 inhibitors have been reported to date, just a few have demonstrated good activity in terms of antitumor cell effect, and practically none of them is near clinical development (Yue and Turkson 2009). Consequently, it is still too soon to predict how patients will benefit from STAT3 inhibitors as anticancer agents, and new efforts on the targeting of the many sites of the STAT3 activation process shall probably arise in the near future.

In the field of reproduction, LIF and the JAK/STAT signaling pathway also deserve some comments. As previously cited in this work, LIF is regarded as a central factor regulating embryo implantation, both in rodents and primates, inasmuch as it stimulates anchorage of the trophoblast, in a process comprising cell adhesion and invasion (Dimitriadis et al. 2010, Singh et al. 2011). The observation that women suffering from repeated implantation failure and unexplained abortion present mutation in the *LIF* gene (Steck et al. 2004) prompted scientists to develop therapies to restore LIF levels during the implantation window. In fact, the importance of LIF for implantation in animal models is highlighted by the observation that embryos fail to implant in *Lif*-deficient female mice, and implantation can be restored after LIF supplementation (Stewart 1994). Additionally, it was recently demonstrated that women have greater chance of getting pregnant when they present strong LIF immunoreactivity

during the implantation phase (Serafini et al. 2009). In this context, the use of recombinant human LIF (r-hLIF) has been tested in preclinical and clinical trials in women with recurrent implantation failure and undergoing *in vitro* fertilization. It could be expected that r-hLIF administered SC might counterbalance the possibly weaker expression of endometrial LIF in these women, with consequent improvement in implantation rates. Unfortunately, a recent multicenter study testing this hypothesis demonstrated discouraging results, with lower clinical pregnancy rates observed in women receiving r-hLIF than in those receiving placebo (Brinsden et al. 2009). In the same study, the authors emphasize the difficulty in recruiting patients with LIF dysregulation, since there is neither a validated tool to assess LIF expression, nor an endometrial LIF threshold level below which there is a correlation with recurrent implantation failure. The overcome of such limitations, with the establishment of normal and pathological threshold levels of LIF expression, shall facilitate the development of new therapies targeting LIF-related implantation disorders.

On the other hand, maintaining the endometrium in a non-receptive state has also been goal of studies, particularly of those concerning the development of novel contraceptive therapies, in which critical factors governing endometrial receptivity are intended to be targeted (Dimitriadis et al. 2010). In these terms, nonhormonal LIF inhibitors have been tested in mice, with consequent prevention of implantation through the targeting of LIF action in endometrial luminal epithelial cells (White et al. 2007). Nevertheless, these results still need to be validated in primates.

In conclusion, one can say that LIF and its underlying JAK/STAT signaling pathway have been intensively investigated over the last years, but a lot of effort is still necessary to completely understand the role of these factors in cell signaling. Many members of this pathway have been demonstrated to be diffusely distributed in the organism and are involved in the transmission of a vast array of signals governing central functions, both in the physiological and pathological settings. The understanding of the role of these factors shall ultimately contribute to the development of therapies targeting cancer and pregnancy-related disorders. We hope that the experiments and results of this work have contributed to better comprehend some aspects of these multifunctional and complex signaling mechanisms.

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Figure Sources

Fig 01. Implanting blastocyst (Figure from Staun-Ram and Shalev 2005)
Fig 02. JAK/STAT signal transduction pathway (Modified from Yamaoka 2004)
Fig 08. Mechanism of RNA interference (Modified from Dykxhoorn 2003)
All figures not cited above are private photos / images.

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Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass

- mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

- ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

- mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben:

  Prof. Dr. med. habil. Udo R. Markert

- die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

- ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

- ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, den 09. September 2013

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André Luís Laranjeira Forti

Equipment, Material and Reagent Specifications

Equipments

Autoclave	KSG-112-Olching
Balance	
Basic	Satorius
Blot Apparatus	Biometra
Centrifuges	
Biofuge 13	Heraeus
Labofuge T	Heraeus
Mikro 22 R	Hettich
Universal 30 F	Hettich
Counting Chamber	Optik Labor
Electrophoresis Chamber	PEQLAB Biotechnology GmbH
Film Cassette	Kisker
Flow Cytometer	BD
FACSCalibur	
Fluorescence Microscope	Zeiss
Axioplan 2	
Gel Documentation System	Biostep GmbH
MF-ChemiBIS 3.2	
Heating Plate MR 3001	Heidolph
Incubator	Heraeus
Hera Cell	
Laminar Airflow Cabinet Steril GARD Hood	Baker Company
Microscope	Zeiss
Axiovert 25	
Orbital Shaker	Heidolph
Polymax 1040	
Pipettes	Eppendorf
Spectrophotometer	Bio Varian
Cary UV 50	

Thermomixer Comfort	Eppendorf
Vortex-Genie 2	Scientific Industries

Materials

Cell Culture Flask	
50 ml, 175 cm^2	Greiner
Cell Culture Plates	
6, 24-well plates	Greiner
Cuvettes	Greiner
Eppendorf Tubes	Sarstedt
0.5, 1.5, 2.0 ml	
Falcon Tubes	Greiner
15 ml, 50 ml	
Film	
Hyperfilm	Amersham Pharmacia Biotech
MIN- R 2000	Kodak
Filter	
Ultrafree-15	Millipore
PVDF Membrane	Amersham Pharmacia Biotech
Hybond-C Extra	

Chemicals and Reagents

1,4-Dithiothreitol (DTT)	Roth
5x siRNA Annealing Buffer	Ambion
Acetic Acid	Roth
Ammonium Persulfate (APS)	Roth
Bis-/Acrylamide (AA/BAA)	Roth
Bromophenol Blue	Roth
Chemiluminescent Substrate	
LumiGLO	Cell Signaling Technology
Carboxyfluorescein Succinimidyl Ester (CFSE)	
Cell Lyses Buffer	Cell Signaling Technology

Developer and Fixer Solutions	Kodak
DMEM	Bio Whittaker
Electrolytic Buffer	Peqlab
Ethanol	Roth
FACS Clean	BD
FACS Flow	BD
FACS Lysing	BD
FACS Rinse	BD
GBSS	Sigma
Glycerin	Roth
Goat Serum	DAKO
Lymphocyte Separation Medium	PAA
Matrigel	BD
Methanol	Roth
Molecular Weight Marker	
Prestained Protein Marker	BioLabs
Precision Plus Protein™ Standards	BIO-RAD
Nanofectin Diluent	PAA
Nuclease Free Water	Ambion
Sodium Chloride (NaCl)	Roth
Sodium Dodecyl Sulphate (SDS)	Roth
Sodium Hydroxide (NaOH)	J.T. Baker
Oligofectamine™ Reagent	Invitrogen
Optimem	Bio Whittaker
Paraformaldehyde	Sigma
PBS	Biochrom AG
Penicillin/Streptomycin	PAA
Poly-L-Lysin	Sigma
Ponceau S	Roth
Protein-Assay	BIO-RAD
Proteinase Inhibitor Cocktail	Sigma
RPMI 1640	PAA
TEMED	Pharmacia Biotech
Tris-Base	Sigma

Tris-HCL	Sigma
Trypan Blue	Sigma
Trypsin/EDTA	Lonza
TWEEN 20	Roth
Zymed Blocking Solution	Invitrogen

Antibodies

Rabbit anti-human ß-Actin	Cell Signaling Technology
Anti-rabbit IgG	Cell Signaling Technology
Goat anti-rabbit IgG-Cy3	Dianova

Oligonucleotide Sequences

JAK1 siRNA Sequences (Eurogentec):

Sense:	5’ GCA GGU GGC UGU UAA AUC 3’ U99
Antisense:	5’ AGA UUU AAC AGC CAC CUG 3’ C99

Non-genomic Oligonucleotide Sequences:

Stat3 102 (Control):

Sense:	5’ GCC ACU UAU AAA UUC GUU Ctt 3’
Antisense:	5’ GAA CGA AUU UAU AAG UGG Ctt 3’

Buffers, Solutions and Gel Composition

10 x Running Buffer:

0,24 M	Tris-Base	
1,9 M	Glycine	
0,1%	SDS	pH 8,5

10 x Tris-Glycine-Buffer:

0,12 M	Tris-Base

0,96 M	Glycine	

Transference Buffer:

1 x	Tris-Glycine-Buffer	
20%	Methanol	

5 x Loading Buffer:

50 mM	Tris-HCL	
2%	SDS	
0,002%	Bromphenol Blue	
0,1 M	DTT	
10% (w/v)	Glycerin	

4 x Lower Tris:

1,5 M	Tris-Base	
0,4%	SDS	pH 8,8

4 x Upper Tris:

0,5 M	Tris-HCL	
0,4%	SDS	

Ponceaus-S-Staining Solution:

0,1%	Ponceau S	
5%	Acetic Acid	

Ponceau-S-Decolourising Agent:

0,8% (w/v)	NaOH	

PBS Tween 20 (Wash Buffer)

2,4g	Tris-Base	
9,0g	NaCl	
0,1%	Tween 20	pH 7,4

PBS 10x (1M) for Immunocytochemistry

87,9g	NaCl	
2,74g	KH2PO4	
11,35g	Na2HPO4	pH 7,2

PBS/tween 20 (0,03%) for Immunocytochemistry

75µl tween20 in 25ml PBS 0,1M